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FHL2 interacts with EGFR to promote glioblastoma growth

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Abstract

Four-and-a-half LIM protein2 (FHL2) is a member of the LIM-only protein family, which plays a critical role in tumorigenesis. We previously reported that FHL2 is upregulated and plays an oncogenic role in glioblastoma (GBM), the most common and aggressive brain tumor. GBM is also marked by amplification of the epidermal growth factor receptor (*EGFR*) gene and its mutations, of which EGFRvIII is the most common and functionally significant. Here we report that FHL2 physically interacts with the wild-type EGFR and its mutated EGFRvIII form in GBM cells. Expression of FHL2 caused increased EGFR and EGFRvIII protein levels and this was due to an increase in protein stability rather than an increase in EGFR mRNA expression. In contrast, FHL2 knockdown using RNA interference reduced EGFR and EGFRvIII protein expression and the phosphorylation levels of EGFR and AKT. Consistent with these features, EGFR expression was significantly lower in mouse FHL2-null astrocytes, where reintroduction of FHL2 was able to restore EGFR levels. Using established GBM cell lines and patient-derived neurosphere lines, FHL2 silencing markedly induced cell apoptosis in EGFRvIII-positive cells. Targeting FHL2 significantly prevented EGFRvIII-positive GBM tumor growth in vivo. FHL2 expression also positively correlated with EGFR expression in GBM samples from patients. Taken together, our

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Compliance with ethical standards

Conflict of interest The authors declare that they have no competing interests.

results demonstrate that FHL2 interacts with EGFR and EGFRvIII to increase their levels and this promotes glioma growth, representing a novel mechanism that may be therapeutically targetable.

Introduction

Glioblastoma (GBM) is the most common and deadly brain tumor in adults. GBM is characterized by its infiltrative growth, necrosis, and highly angiogenesis nature, which hamper the effectiveness of current standard therapy with surgery, radiotherapy and temozolomide chemotherapy. As a result, GBM almost always recurs and the patients have a median survival of roughly 16 months, with a 5-year survival rate of only 3–5% [1].

Four-and-a-half LIM protein 2 (FHL2) is a member of the LIM-domain-only protein family [2]. FHL2 is involved in the development and progression of various types of carcinoma. The function of FHL2 in tumorigenesis is context-dependent, as it functions as an oncogene in breast, gastrointestinal, ovarian, and cervical cancers [3–6], but acts as a tumor suppressor in hepatocellular carcinoma and rhabdomyosarcoma [7–9]. FHL2 is known to interact with >50 different binding partners through its LIM domains, suggesting why modifying the its expression might result in varying downstream effects depending on cell type. Furthermore, the zinc-finger motifs in the LIM domains of FHL2 confer function as a transcriptional co-activator/repressor in various cancers [10].

Some of us previously reported that FHL2 plays a critical role in glioma [11]. We demonstrated that FHL2 is highly expressed in both glioma cell lines and human glioma specimens. Silencing FHL2 with RNA interference inhibits glioblastoma cell growth and migration. Conversely, upregulating FHL2 expression in glioma cell lines stimulates proliferation, anchorage-independent growth, migration, and tumorigenicity in mice. It can also increase the promoter activities of AP-1, human telomerase reverse transcriptase (hTERT), and survivin genes in GBM [12]. In line with our findings, The Cancer Genome Atlas (TCGA) dataset showed that FHL2 is one of the top 1000 dysregulated genes in GBM [12].

The epidermal growth factor receptor *(EGFR*) is mutated, amplified, or both in nearly 60% of GBMs, and EGFRvIII mutation frequency is 20–30% [13]. EGFRvIII consists of an in-frame exon 2–7 deletion that leads to the constitutive activation of downstream signaling [13]. EGFRvIII is a signature marker for the classical subtype of GBM, and it plays a key role in GBM growth and invasion. EGFRvIII is considered as a therapeutic target, given its tumor-specific expression pattern and its prevalence in GBM [14, 15]. However, clinically targeting EGFRvIII using small molecule inhibitors or antibodies has not demonstrated durable responses. Therefore, identifying novel approach(s) to treat EGFRvIII-positive GBM is needed.

Here we investigated whether there is a relationship between FHL2 and EGFR in GBM. We demonstrate that FHL2 physically interacts with EGFR and EGFRvIII to positively regulate their protein stability in GBM cells. *FHL2* silencing by RNA interference dramatically reduces EGFR and EGFRvIII protein levels and inhibits downstream PI3K-AKT pathway. Moreover, FHL2 knockdown significantly inhibits EGFRvIII-promoted cell proliferation in

vitro and in vivo. These results suggest that FHL2 might be a novel potential therapeutic target for EGFRvIII positive GBM.

Results

FHL2 interacts with EGFR and EGFRvIII in glioma cells

We first determined the relationship between FHL2 and EGFR expression in GBM patient samples. As shown in Supplementary Fig. 1A, protein levels of both FHL2 and EGFR are highly elevated in GBM tumors as compared to normal brain tissue. Interestingly, the expression of FHL2 and EGFR displayed a significant positive correlation pattern (Supplementary Fig. 1B, Pearson $r = 0.6338$, $p = 0.0362$). A previous report using mass spectrometry demonstrated that FHL2 might be one of the interacting partners of EGFR in the human squamous carcinoma line, HN5 [16]. We, therefore, examined whether FHL2 is able to associate with EGFR in glioma cells. Co-immunoprecipitation (co-IP) assay was performed using U87MG cells, which express low levels of endogenous EGFR and FHL2, and found that EGFR physically interacted with FHL2 (Fig. 1a). We then determined whether EGFRvIII, the common mutated EGFR form lacking a portion of the extracellular ligand-binding domain, can interact with FHL2 in glioma cells. We performed co-IP using patient-derived neurosphere lines GSC-1, which expresses endogenous EGFRvIII and FHL2, and showed that FHL2 associated with EGFRvIII (Fig. 1b). We also did co-IP using U87-EGFRvIII cells, which were transfected with PCI-3xFlag-FHL2. As shown in Fig. 1c, EGFRvIII was detected in the immunoprecipitates captured by anti-Flag antibody in U87-EGFRvIII cells (upper panel). Conversely, FHL2 was captured in the anti-EGFRvIII immunoprecipitates (lower panel). We then mapped the potential interaction domains of FHL2 that interact with EGFRvIII. To this end, we separately transfected four different PCI-3xFlag-tagged plasmids (encoding the full-length and three truncated mutants of FHL2, termed 76, 77, 78, and 79, respectively) into U87-EGFRvIII cells and then performed co-IP. It was shown that only the full-length form of FHL2 was able to interact with EGFRvIII, while the three truncated mutants of FHL2 failed to do so (Fig. 1d), suggesting that at least the half LIM and LIM1 domains are essential for the interaction. Moreover, immunofluorescence confocal microscopy further confirmed that FHL2 and EGFRvIII interacted with each other and co-localized primarily in the cytoplasm of U87-EGFRvIII cells (Fig. 1e).

FHL2 regulates EGFR and EGFRvIII protein expression in glioma cells

Since FHL2 is associated with EGFR and EGFRvIII, we next asked if it is involved in regulating EGFR-driven signaling. We introduced FHL2 into U87MG and U87-EGFRvIII cells, and surprisingly found that overexpression of FHL2 markedly increased both EGFR and EGFRvIII protein levels and downstream AKT phosphorylation in both cells (Fig. 2a, b, and Supplementary Fig. 2). Consistent with this, FHL2 silencing using RNAi reduced EGFRvIII protein levels and phospho-AKT levels in U87-EGFRvIII cells (Fig. 2c). FHL2 knockdown also reduced endogenous EGFRvIII protein levels in GSC-1 cells (Fig. 2d). In contrast, EGFR or EGFRvIII depletion with RNAi failed to affect FHL2 expression in U87-MG, U87-EGFRvIII and two independent GBM neurosphere cells, GSC-1 and GSC-2 (Fig. 2e).

Using U87-wtEGFR cells that ectopically overexpress wild-type EGFR and SF767 cells that express high levels of endogenous EGFR, FHL2 expression was suppressed using RNAi with or without EGF stimulation. This FHL2 knockdown significantly reduced EGFR protein expression in both U87-wtEGFR (high exogenous EGFR expression) and SF767 (high endogenous EGFR expression) cells. As expected, EGF-stimulated EGFR activation, and downstream phospho-AKT levels were also decreased when FHL2 was depleted with RNAi (Supplementary Fig. 3). To further verify the relationship between FHL2 and EGFR, we isolated astroctyes from FHL2$^{+/+}$ and FHL2$^{-/-}$ BALB/c mice (3 of each) and analyzed EGFR and FHL2 expression by immunobloting. EGFR protein levels were significantly lower in FHL2 knockout cells compared to those in wild-type astrocytes (Fig. 2f). Moreover, EGFR was not detectable in a spontaneously immortalized FHL2 null P26M MEF cell line [17]. When FHL2 was reintroduced into the FHL2$^{-/-}$ MEF cells, EGFR expression was restored (Fig. 2g). Collectively, these data strongly suggested that FHL2 is able to regulate EGFR and EGFRvIII protein expression.

FHL2 protects EGFR and EGFRvIII from proteasome-mediated degradation

Next we determined how FHL2 regulates EGFR and EGFRvIII expression. We first examined if FHL2 affects *EGFR* mRNA transcription or stability. FHL2 overexpression failed to affect endogenous EGFR mRNA levels in U87MG cells (Fig. 3a). To further explore this, we transfected an EGFR promoter reporter, pER1-luc, containing the 1109 bp 5′-untranscribed region of the EGFR promoter which can be activated by Notch-1 signaling [18] (Supplementary Fig. 4), into U87MG cells and examined if FHL2 could influence EGFR promoter activation. Luciferase assays showed that the EGFR promoter activity was not significantly affected by ectopic FHL2 expression in U87MG cells (Fig. 3b) under conditions where the reporter could be activated by a Notch-1 construct. Consistent with this result, there were no obvious changes in the mRNA levels of *EGFR* when U87 parental cells were transfected with two different lentiviral FHL2 shRNAs (Fig. 3c). Similarly, no obvious changes in the mRNA levels of endogenous EGFR and exogenous EGFRvIII were observed when FHL2 was silenced in U87-EGFRvIIIl cells (Fig. 3d).

It has been reported that many LIM-domain proteins could affect proteasome-mediated protein degradation through interactions with E3 ligases, and FHL2 has been shown to be a target for Murf3 (an E3 ligase) [19]. Therefore, we tested whether FHL2 influences EGFR and EGFRvIII protein stability. U87MG or U87-EGFRvIII cells were separately transfected with pBabe-FHL2 or control plasmid (V), and the cells were then treated for 24 h with 20 μM MG-132, a proteasome inhibitor. As shown in Fig. 3e, g, MG-132 treatment was able to increase EGFR and EGFRvIII protein levels in the control cells (lane 1 vs. 3). FHL2 overexpression alone increased EGFR and EGFRvIII expression (lane 1 vs. 2). Of note, overexpression of FHL2 led to a further increase in EGFR and EGFRvIII levels in the MG-132-treated cells (lane 2 vs. 4). Consistent with this data, FHL2 silencing with two different lentiviral shRNAs led to a further reduction in EGFR and EGFRvIII protein levels in the MG-132-treated U87MG and U87-EGFRvIII cells (Fig. 3f, h). Taken together, these data strongly suggest that FHL2 is able to regulate EGFR and EGFRvIII protein stability.

FHL2 is essential for EGFRvIII-mediated GBM cell proliferation in vitro

Given the important oncogenic role of the EGFRvIII mutant form in GBM, we next assessed the potential role of FHL2 in EGFRvIII-promoted GBM cell growth. *FHL2* was silenced by two different lentiviral shRNAs in U87-EGFRvIII cells (Fig. 4a). WST-1 assays showed that FHL2 knockdown markedly inhibited EGFRvIII-stimulated cell proliferation (Fig. 4b). In contrast, overexpression of FHL2 alone increased U87 cell growth when compared to the vector control. Additionally, FHL2 overexpression further enhanced U87-EGFRvIII cell proliferation (Fig. 4c). Consistent with this, FHL2 silencing significantly inhibited EGFRvIII-promoted cell growth in two independent patient-derived neurosphere lines GSC-1 and GSC-2 (both express high levels of endogenous EGFRvIII) (Fig. 4d–f). These results suggest that FHL2 plays a key role in EGFRvIII-mediated GBM cell growth.

FHL2 silencing induces cell apoptosis in EGFRvIII-expressing GBM cells

We next investigated the mechanism(s) by which FHL2 silencing inhibited EGFRvIII-promoted cell proliferation. PI/AnnexinV staining showed that FHL2 knockdown significantly increased cell death at the late stage (shGFP: 0.67 ± 0.18%, shFHL2#1: 4.41 ± 0.7%, $p < 0.01$; shFHL2#4: 4.16 ± 0.64%, $p < 0.05$. Fig. 5a, b). Similar results were obtained in GSC-1 and GSC-2 cells where FHL2 silencing significantly induced late apoptosis (Fig. 5c, d and Supplementary Fig. S5). Consistent with this, the apoptosis markers, Bax and cleaved PARP, were increased when FHL2 expression was depleted in U87-EGFRvIII or in GSC-1 cells (Fig. 5e). Interestingly, in the absence of EGFRvIII, FHL2 knockdown significantly induced cell cycle arrest in G0/G1 phase but not cell apoptosis in U87MG cells (Supplementary Fig. S6). Collectively, these data suggest that FHL2 interaction with EGFRvIII promotes GBM cell viability in part by reducing cell apoptosis.

FHL2 is required for EGFRvIII-mediated tumor growth in vivo

Finally, we determined whether *FHL2* knockdown could inhibit the in vivo growth of EGFRvIII-expressing GBM cells. U87-EGFRvIII-shGFP, U87-EGFRvIII-shFHL2#1, and U87-EGFRvIII-shFHL2#4 cells were injected subcutaneously into BALB/c-nu/nu mice. We found that *FHL2* knockdown significantly reduced tumor growth rates compared to the control group ($p < 0.01$, Fig. 6a). U87-EGFRvIII-shGFP, U87-EGFRvIII-shFHL2#1, and U87-EGFRvIII-shFHL2#4 cells were also orthotopically implanted into the brains of nude mice. Microscopic analysis of brain sections showed that the mice implanted with U87-EGFRvIII-shGFP cells (the control group) developed very large tumors, whereas mice implanted with shFHL2 cells developed smaller tumors (Fig. 6b). Immunohisto-chemical (IHC) staining showed that *FHL2* knockdown decreased FHL2 and EGFRvIII protein expression, but increased the number of TUNEL-positive cells (2.9 fold shFHL2#1 vs. shGFP, $p < 0.05$, and 3.0-fold shFHL2#4 vs. shGFP, $p < 0.01$) and of Caspase-3-positive cells (2.8 fold shFHL2#1 vs. shGFP, 2.4 fold, $p < 0.05$, and shFHL2#4 vs. shGFP, $p < 0.05$) (Fig. 6c–e). Taken together, these data suggest that FHL2 increases EGFRvIII-mediated tumor growth in part through reducing cell apoptosis.

Discussion

Here we provide the first evidence that FHL2 as an interacting partner of EGFR and EGFRvIII, increasing their protein stability and consequently increasing GBM tumorigenicity. We show that *FHL2* silencing using RNA interference significantly reduced EGFR and EGFRvIII expression at the protein level but not at the mRNA level. Functionally, FHL2 knockdown inhibited EGFRvIII-expressing GBM cell growth through induction of cellular apoptosis in vitro and in mice. *FHL2* silencing caused delayed EGFRvIII-expressing GBM tumor progression. Importantly, we showed that FHL2 expression is positively correlated with EGFR expression in GBM patients. Therefore, targeting FHL2 might be a novel strategy to treat EGFRvIII-positive GBM.

EGFRvIII, lacking exons 2–7, is the most common mutated form of EGFR in GBM. EGFRvIII plays a key role in gliomagenesis mainly through its constitutive phosphorylation and downstream activation of the Ras/MAPK and PI3K-AKT pathways [20]. Like wtEGFR, EGFRvIII is mainly localized to the cell membrane. However, numerous reports have demonstrated that, under pathological and some stress conditions, EGFR and EGFRvIII can translocate to the nucleus, where they interact with transcription factors such as Stat3 and Stat5 to regulate gene expression [21–23]. In the present study, we show that FHL2 associates with EGFRvIII mainly in the cytoplasm and functions as a stabilizer of EGFRvIII protein, which in turn promotes cell proliferation through the PI3K-AKT pathway in part and reducing cell apoptosis. Although FHL2 can act as a transcriptional co-factor, it seems unlikely that FHL2 interacts with EGFRvIII in the nucleus to regulate target gene expression in this case.

It has been well established that the function of FHL2 is cell type dependent. This is perhaps because FHL2 is able to interact with multiple proteins via its LIM domains, and the expression pattern of the interacting partners is different. We previously reported that FHL2 is upregulated and plays an oncogenic role in GBM [11]. However, the molecular mechanism by which FHL2 promotes GBM invasion and tumor growth remained elusive. Our present study shows that FHL2 alone increased GBM cell proliferation, and FHL2 overexpression was able to further promote EGFRvIII-mediated cell growth. On the other hand, *FHL2* knockdown significantly inhibited EGFRvIII-promoted GBM cell growth. Given the fact that FHL2 can enhance EGFRvIII protein expression and the important oncogenic role of EGFRvIII in GBM, we propose that FHL2 promotes GBM tumor growth and progression through partially stabilizing EGFRvIII and promoting downstream signaling pathways such as PI3K-AKT without changing the total AKT level. Interestingly, a recent report has demonstrated that FHL2 directly regulates AKT1 expression in ovarian granulosa cell tumor [5].

In GBM, wtEGFR, and EGFRvIII have been shown to interact with numerous proteins. For instance, the extracellular matrix molecule integrin β3 binds with EGFRvIII to promote GBM progression and metastasis in hypoxic and vitronectin-enriching microenvironment [24]. The EGF-activated Axl Receptor tyrosine kinase (RTK) forms a heterodimer with EGFR (Axl-EGFR) to promote GBM invasion by inducing MMP9 expression [25]. Another important RTK c-Met can associate with both wtEGFR and EGFRvIII. A recent report

showed that EGF-stimulated EGFR activation leads to a dissociation of the EGFRvIII–Met complex with a concomitant loss of Met phosphorylation [26]. The cytokine receptor OSMR can form a co-receptor with EGFRvIII to activate STAT3 to promote GBM tumor growth. Interestingly, OSMR itself is a direct target of EGFRvIII-STAT3 pathway [27]. Several studies have reported that both EGFR and EGFRvIII can also translocate to mitochondria, which is involved in tumor drug resistance [28, 29]. It has been shown that PUMA (53-upregulated modulator of apoptosis) interacts with EGFR and EGFRvIII to inhibit mitochondrial translocalization of PUMA and PUMA-induced apoptosis [29].

It has been reported that EGFR stability plays a role in the survival of EGFR-driven cancers. Leucine-rich repeat and immunoglobulin-like domain protein-1 (LRIG1) is shown to interact with and destabilize EGFRvIII to negatively regulate its oncogenic activity [30]. Mig-6 can interact with both wtEGFR and EGFRvIII. However, it can only promote wtEGFR into late endosome and lysosome-mediated degradation upon ligand stimulation because EGFRvIII rarely undergoes ligand-induced internalization and the subsequent lysosome-mediated degradation. Our study demonstrated that FHL2 interacted with and stabilized wtEGFR and EGFRvIII, and the interaction is independent of EGFR activation since a kinase-dead mutant of EGFR-vIII (DK) can also bind to FHL2 (Supplementary Fig. 7). Both wtEGFR and EGFRvIII can undergo activation-dependent downregulation mediated by the family of Cbl ubiquitin ligases [31]. As a LIM-domain-containing protein, FHL2 is a target for Murf3 (an E3 ligase) [17] in muscle. It has been reported that other LIM-domain proteins could inhibit proteasome-mediated degradation by interacting with E3 ligases or ubiquitin targets [32, 33]. The current study showed that overexpression of FHL2 protected proteasome-mediated protein degradation of EGFR and EGFRvIII by using proteasome inhibitor MG132 (Fig. 3). Therefore, we speculate that FHL2 may function as a stabilizing cofactor that prevent binding of ubiquitin ligases to EGFR or EGFRvIII, which in turn prevent protein degradation of these two proteins. However, the detailed mechanism of the regulating ubiquitination of EGFR/EGFRvIII needs to be further explored.

Although EGFRvIII plays an oncogenic role in GBM, EGFRvIII by itself is not sufficient to transform glial cells. Other molecules such as Ras and PI3K/AKT are also needed to induce glioma in mouse and *Drosophila* models [34–36]. FHL2 has been shown to play an essential role for Ras-induced transformation through the cyclin D1 and p53 pathways using MEF cells as a model [37]. Our current study provides additional evidence that FHL2 physically interacts with EGFRvIII through its LIM domains to stabilize EGFRvIII, which in turn, further activates downstream signaling cascades such as the PI3K-AKT pathway. We further showed that FHL2 silencing using RNAi significantly reduced EGFRvIII expression in vitro and in tumor. Importantly, FHL2 knockdown significantly inhibited EGFRvIII + tumor growth in mice, indicating that inhibiting FHL2 might provide an alternative approach to targeting EGFRvIII-positive GBM.

In summary, our results demonstrate, to our knowledge, for the first time that FHL2 associates with EGFR and EGFRvIII to increase their protein levels and downstream signaling pathway activation, promoting GBM tumor growth. Our data suggest that targeting FHL2 might be a novel approach to treat GBM with EGFRvIII expression.

Materials and methods

Cell lines

GBM cell lines U87MG and SF767 were cultured in DMEM medium with 10% FBS. U87-EGFRvIII cells were maintained in DMEM/10% FBS medium with G418 (400 ng/ml) [38]. Two primary GBM patient-derived neurosphere lines GSC-1 and GSC-2 (both express high levels of endogenous EGFRvIII) were isolated from untreated GBM (WHO IV) patients after surgery at the Second Affiliated Hospital of Soochow University with the patient's approval, then maintained in DMEM/F12K medium supplemented with N-2 (Invitrogen), B27 (Invitrogen), glutamine, EGF (25 ng/ml), and bFGF (25 ng/ml). P26M FHL2 null MEF cells were obtained from Dr. Yu Wei, and were maintained in DMEM/10% FBS as described previously [27]. Astrocytes were isolated from brains of wild-type and FHL2 knockout C57BL/6 J postnatal (P3) pups [39]. The isolation and culture of the mouse astrocytes were described previously [40].

Constructs and siRNAs

To map the interaction domains, four FLAG-tagged constructs 76, 77, 78, and 79 were cloned in PCI-3xFlag plasmids expressing the full-length, the last three, two, and one LIM domain of FHL2 protein were described previously [41]. pLKO.1-shGFP (5′-CAAGCTGACCCTGAAGTTCAT-3′), pLKO.1-shFHL2#1 (5′-GAGACTTTCTTCTAGTGCTTTC-3′) or pLKO.1-shFHL2#4 (5′-CGAATCTCTCTTTGGCAAGAAC-3′) was obtained from Sigma. pBabe-puro-FHL2 was subcloned from PCI-3xFlag-FHL2 as described previously [37]. pER1-luc, the EGFR promoter luciferase reporter plasmids containing a fragment of 1109 bp of EGFR promoter was described previously [42]. siRNAs against FHL2, EGFR and negative control were purchased from Santa Cruz Biotech. siRNA transfection was performed using Lipofectamine RNAiMAX Reagent (Invitrogen).

Virus production and infection

Lentiviral shRNAs (pLKO.1-shGFP, pLKO.1-shFHL2#1, and pLKO.1-shFHL2#4) were produced in 293FT cells, which were cotransfected by with pCMVDR8.91 and pMD. G-VSVG using Lipofectamine 3000 (Invitrogen). To make retrovirus, 293FT cells were transfected with pBabe-puro-LacZ or pBabe-puro-FHL2 and pCL10A1. All viral supernatants were cleaned up with a 0.45 μm filter at 48 and 72 h posttransfection. Glioma cells were infected overnight with virus in the presence of polybrene (8 μg/ml). After 2 days of infection, the infected cells were selected for 5–7 days with 1 μg/ml puromycin to establish positive stable clones.

RT-qPCR

Total RNA was reversely transcribed with SuperScript III First Strand kit (Invitrogen) to generate the first strand cDNA, and qPCR was performed using SYBR green PCR MasterMix (ABM) as previously reported [38]. The primers used were as follows: GAPDH forward: 5′-GAAGGTGAAGGTCGGAGTCA-3′, and reverse: 5′-TTGAGGTCAATGAAGGGGTC-3′;

EGFR forward: 5′-GTGCAGATCGCAAAGGTAATCAG-3′, and reverse: 5′-GCAGACCGCATGTGAGGAT-3′; EGFRvIII forward: 5′-GGCTCTGGAGGAAAAGAAAGGTAAT-3′, and reverse: 5′-TCCTCCATCTCATAGCTGTCG-3′.

Immunoblot and antibodies

Cell lysates were produced with NP40 buffer (150 mM NaCl/1.0% Triton X-100/0.5% Na deoxycholate/0.1% SDS/50 mM Tris, pH 8.0) with complete protease inhibitor (Roche) and PMSF (1 mM). Protein concentration was determined using BCA kit (Beyotime). 20 μg of protein samples were separated on SDS-PAGE and transferred to the nitrocellulose membrane using the Bio-Rad gel system. Immunoblots were performed with the following antibodies: anti-EGFR (sc-373746, Santa Cruz Biotech), anti–β-actin (60008-1-Ig, Proteintech), anti-FHL2 (K0055–3, MBL), anti-FLAG (F1804, Sigma), anti-pAKT(Ser473) (4060 s, CST), anti-pEGFR (4267 s, 6963 s, CST), and anti-Phosphotyrosine Antibody clone 4G10® (MAP3090, Upstate).

Immunoprecipitate

Cell lysates were prepared with protein lysate buffer NP40 (Beyotime) plus PMSF (final concentration 1 mM). Protein lysates (500 μg) were incubated with 5 μg/ml of anti-Flag antibody (Sigma), anti-EGFR (SC-03, Santa Cruz Biotech), or isotype control antibody (Santa Cruz Biotechnology) at 4 °C overnight. The mixture was then incubated with Dynabeadsprotein G (10003D, Life) for 4 h at 4°C. The yielded precipitates were analyzed with immunobloting with anti-EGFR (sc-373746, Santa Cruz Biotechnology) and anti-FHL2 (K0055–3, MBL).

Apoptosis analysis

GBM cell line and GBM patient-derived neurosphere lines were stained with Annexin V-FITC/propidium iodide (Vazyme, China) according to the manufacturer’s instructions. Cell apoptosis was analyzed with flow cytometry (Ctoflex, Beckman).

Cell proliferation assay

Cells were seeded into 96-well plates (1.5×10^3 cells per well), and the viable cells were analyzed with WST-1 assay on indicated days. Absorbance was measured at 450 nm on GENios Pro (Tecan) microplate reader.

Xenograft model and histological analysis

Animal studies were approved by the Animal Experimental Committee of Soochow University and performed according to the Ethical Principles and Guidelines of the Second Affiliated Hospital of Soochow University Institutional Animal Care and Use Committee. The 4- to 6-week-old athymic nude mice were randomly grouped and implemented blindingly. For subcutaneous studies, 1×10^6 U87-EGFRvIII-shGFP, U87-EGFRvIII-shFHL2#1, or U87-EGFRvIII-shFHL2#4 cells in 100 μl PBS were inoculated into the flank of mice (8 mice/group). Tumor volume (V) was measured on indicated days with a caliper and calculated using the formula $V = 1/2 \text{ length}^2 \times \text{width}$. For intracranial

studies, 2×10^5 of U87-EGFRvIII-shGFP, U87-EGFRvIII-shFHL2#1, or U87-EGFRvIII-shFHL2#4 cells in 10 μl PBS were injected intracranially into nude mice (6 mice per group) as described by Lan et al [38]. On day 30, mice were killed, and their brains were removed for immunohistochemistry studies. The tumor tissues were sectioned and subjected to hematoxylin and eosin (H&E), anti-EGFR (Santa Cruz Biotech) and anti-FHL2 (MBL) staining. The cell apoptosis in tumor was analyzed with In Situ BrdU-Red DNA Fragmentation kit (TUNEL, Abcam) and anti-Caspase-3 (CST) staining.

Data analysis

All the results are shown as the mean ± SD from at least three independent experiments. Student's two-tailed *t* test was used for statistical analyses. The potential correlation between FHL2 and EGFR expression was analyzed with Pearson correlation coefficient. $p < 0.05$ was considered statistically significant.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgements

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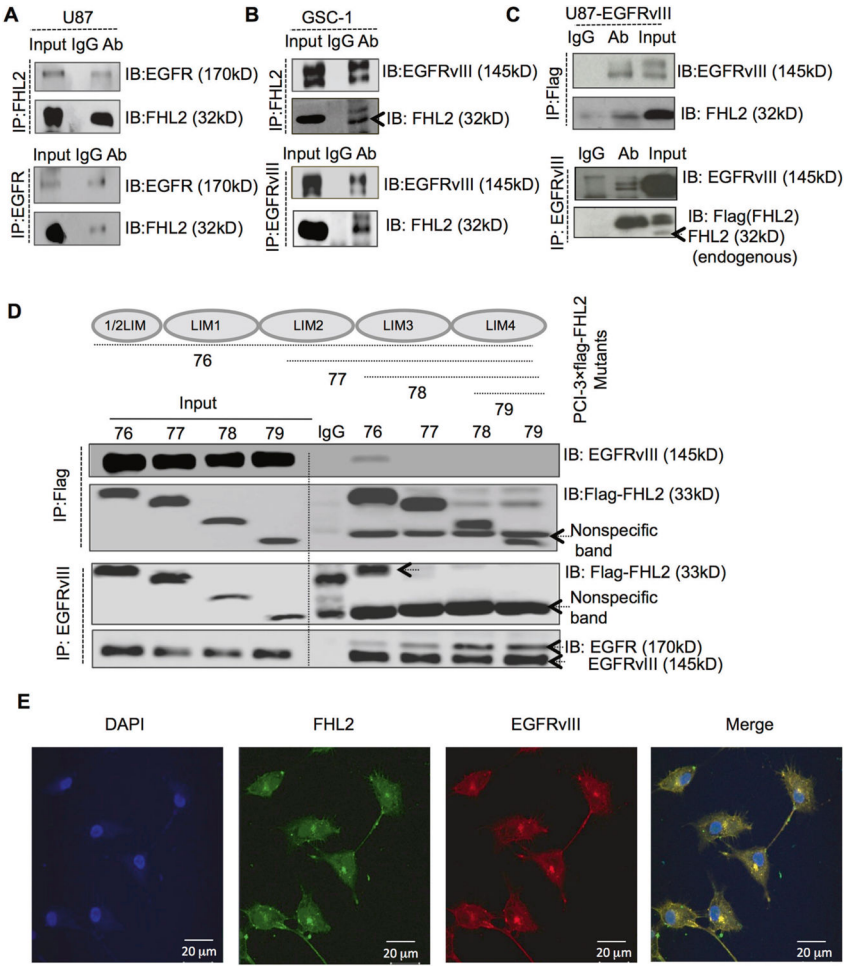

Fig. 1.

FHL2 interacts with EGFR and EGFRvIII in established GBM cell lines and in primary GBM patient-derived neurosphere lines. **a**, **b** Co-Immunoprecipitation was performed with anti-FHL2 antibody (upper panel) or anti-EGFR antibody (lower panel) in cells lysates from U87MG cells (which express endogenous EGFR and FHL2) and GSC-1 cells (which express endogenous EGFRvIII and FHL2). Mouse or Rabbit normal IgG was used as negative control. Immunoblot was performed with indicated antibodies. The whole cell lysates were used as positive controls (Input). **c** U87-EGFRvIII cells were transfected with pCI-3xFlag-FHL2. Immunoprecipitation was performed with anti-Flag antibody (upper panel) or anti-EGFR antibody (lower panel). Mouse or Rabbit normal was used as negative control. Western blotting was performed with indicated antibodies. The whole cell lysates were used as positive controls (Input). **d** Diagram showing the full-length FHL2 (PCI-3 × Flag-76) and the truncations (PCI-3 × Flag-77, 78, 79). IP was performed using the cell lysate of U87-EGFRvIII cells, which was separately transfected with the above four plasmids. Anti-Flag or IgG were used as bait to detect EGFR-vIII (the upper two rows). Anti-EGFR was used as bait to detect FHL2 (the lower two rows). The whole cell lysates were used as positive controls (Input). **e** Visualization of the subcellular localization of FHL2 (green) and EGFRvIII (red) protein in U87-EGFRvIII cells under confocal

fluorescence microscopy. Nuclear DNA was visualized by DAPI staining (left). A merged image of the green/red fluorescence and DAPI staining is also presented (right). The data shown were the representatives of three independent experiments with similar results. IP immunoprecipitation, *IB* immunoblot, *Ab* antibody

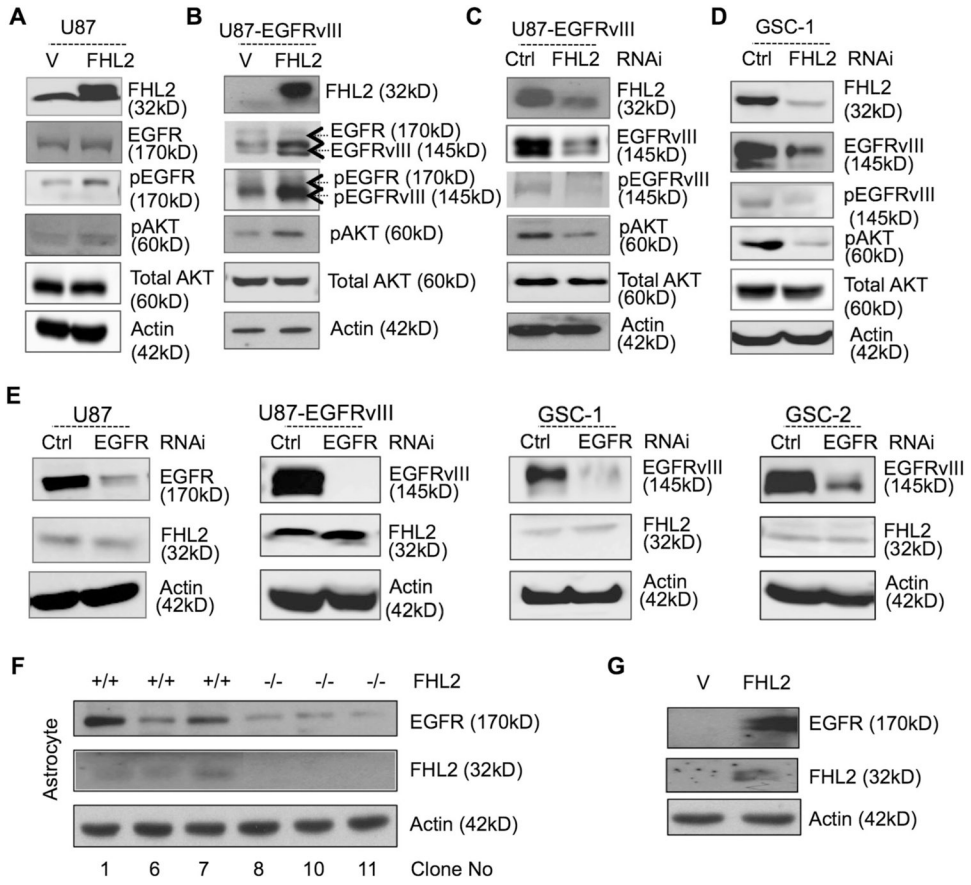

Fig. 2.

FHL2 regulates EGFR and EGFRvIII protein expression. **a**, **b** U87MG (**a**) and U87-EGFRvIII cells (**b**) were infected with pBabe (V) or pBabe-FHL2 retrovirus prior to immunoblot analysis. FHL2, AKT, pAKT, EGFRvIII, and pEGFR(4G10) were analyzed by immunoblot. **c, d** U87-EGFRvIII cells (**c**) and patient-derived neurosphere line GSC-1 (**d**) were transfected with FHL2 siRNA and control siRNA prior to immunoblot. FHL2, AKT, pAKT, EGFRvIII, and pEGFR (4G10) were analyzed by immunoblot. **e** U87 MG, U87-EGFRvIII, GSC-1, and GSC-2 were separately delivered by EGFR siRNA and control siRNA prior to immunoblot analysis of FHL2 and EGFR expression. **f** EGFR and FHL2 immunoblot was performed using astrocyte cell lysates isolated from different clones of wild type ($FHL2^{+/+}$) or $FHL2^{-/-}$ mice. **g** P26M $FHL2^{-/-}$ MEF cells were transfected with pBabe-FHL2 or pBabe (v) prior to immunoblot analysis of EGFR and FHL2 expression. The data shown are representative of three independent experiments with similar results

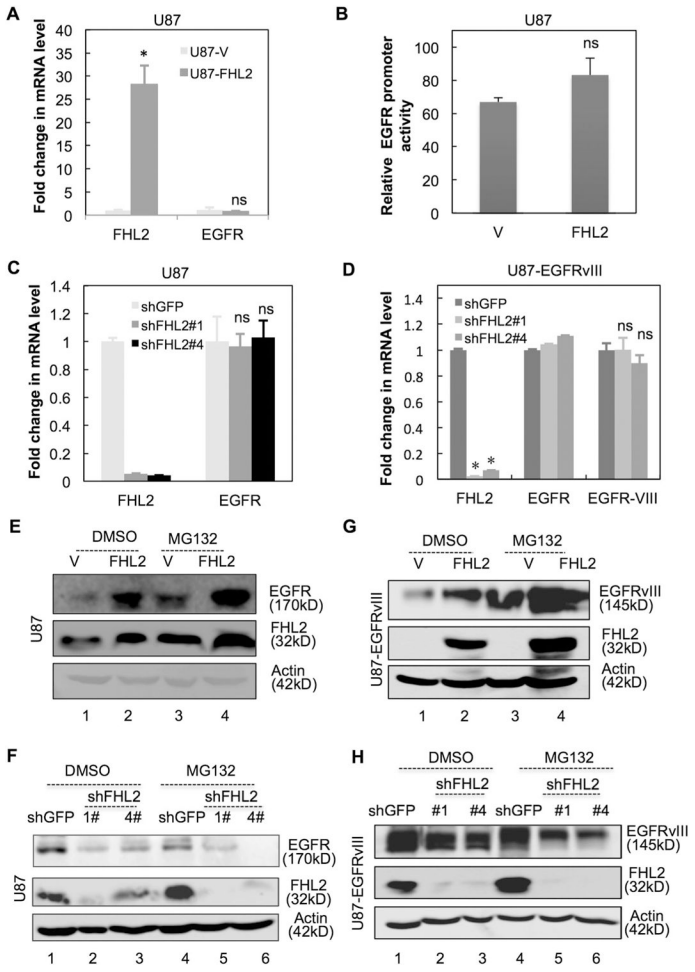

Fig. 3.

FHL2 increases EGFR and EGFRvIII protein stability in vitro. **a** mRNA expression levels of FHL2 and EGFR in U87-pBabe (V) and U87-FHL2 cells were analyzed with RT-qPCR. ns, no statistically significant difference. **b** Effect of FHL2 on pER1-luc (EGFR promoter) activity in U87MG cells was analyzed by luciferase assay. U87MG cells were cotransfected with pcDNA3.1 or pcDNA3.1-FHL2 and pER1-Luc (EGFR promoter) and pRL-TK (internal control) for 24 h prior to the luciferase assay. **c** mRNA expression levels of FHL2 and EGFR in U87-shGFP, U87-shFHL2#1 and U87-shFHL2#4 cells were analyzed with RT-qPCR. $*p < 0.05$. ns, no statistically significant difference. **d** mRNA expression levels of FHL2, EGFR and EGFRvIII in U87-EGFRvIII-shGFP, U87-EGFRvIII-shFHL2#1 and U87-EGFRvIII–shFHL2#4 cells were analyzed with qRT-PCR. $*p < 0.05$. ns, no statistically significant difference. **e** U87-pBabe and U87-FHL2 cells were treated with MG-132 (20 μM) or DMSO for 24 h prior to immunoblot analysis. **f** U87-shGFP, U87-shFHL2#1, and U87-shFHL2#4 cells were treated with MG-132 (20 μM) or DMSO for 24 h prior to immunoblot analysis. **g** U87-EGFRvIII-pBabe and U87-EGFRvIII–FHL2 cells were treated with MG-132 (20 μM) or DMSO for 24 h prior to immunoblot analysis. **h** U87-EGFR-vIII-shGFP, U87-EGFRvIII-shFHL2 #1, and U87-EGFRvIII–shFHL2 #4 cells

were treated with MG-132 (20 μM) or DMSO for 24 h prior to immunoblot analysis. The data shown are representative of three independent experiments

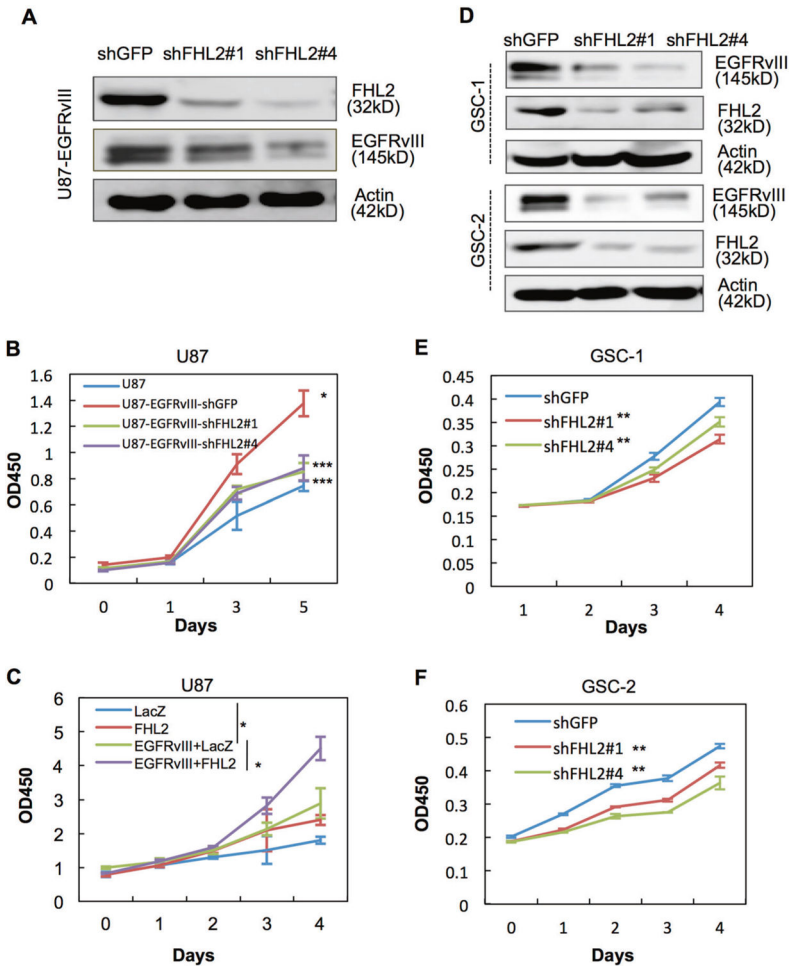

Fig. 4.

FHL2 is essential for EGFRvIII-promoted cell proliferation in vitro. **a** Two independent shRNA sequences (shFHL2#1 and shFHL2#4) were delivered to U87-EGFR-vIII cells with lentiviral vectors pLKO.1. FHL2 and EGFRvIII expression were analyzed with immunoblot. **b** WST1 assay were performed with U87, U87-EGFRvIII-shGFP, U87-EGFR-vIII-shFHL2#1, and U87-EGFRvIII-shFHL2#4 for indicated days. *$p < 0.05$; ***$p < 0.001$. **c** WST1 assay were performed with U87-LacZ, U87-FHL2, U87-EGFRvIII-LacZ, and U87-EGFRvIII-FHL2 cells for the indicated days. *$p < 0.05$. **d** Two independent shRNA sequences (shFHL2#1 and shFHL2#4) were delivered to GSC-1 and GSC-2 cells with lentiviral vectors pLKO.1. FHL2 and EGFRvIII expression were analyzed with immunoblot. **e** WST1 assay were performed with GSC-1-shGFP, GSC-1-shFHL2#1, and GSC-1-shFHL2#4 for indicated days. **$p < 0.01$. **f** WST1 assay were performed with GSC-2-shGFP, GSC-2-shFHL2#1, and GSC-2-shFHL2#4 for indicated days. **$p < 0.01$. The data shown are representative of three independent experiments

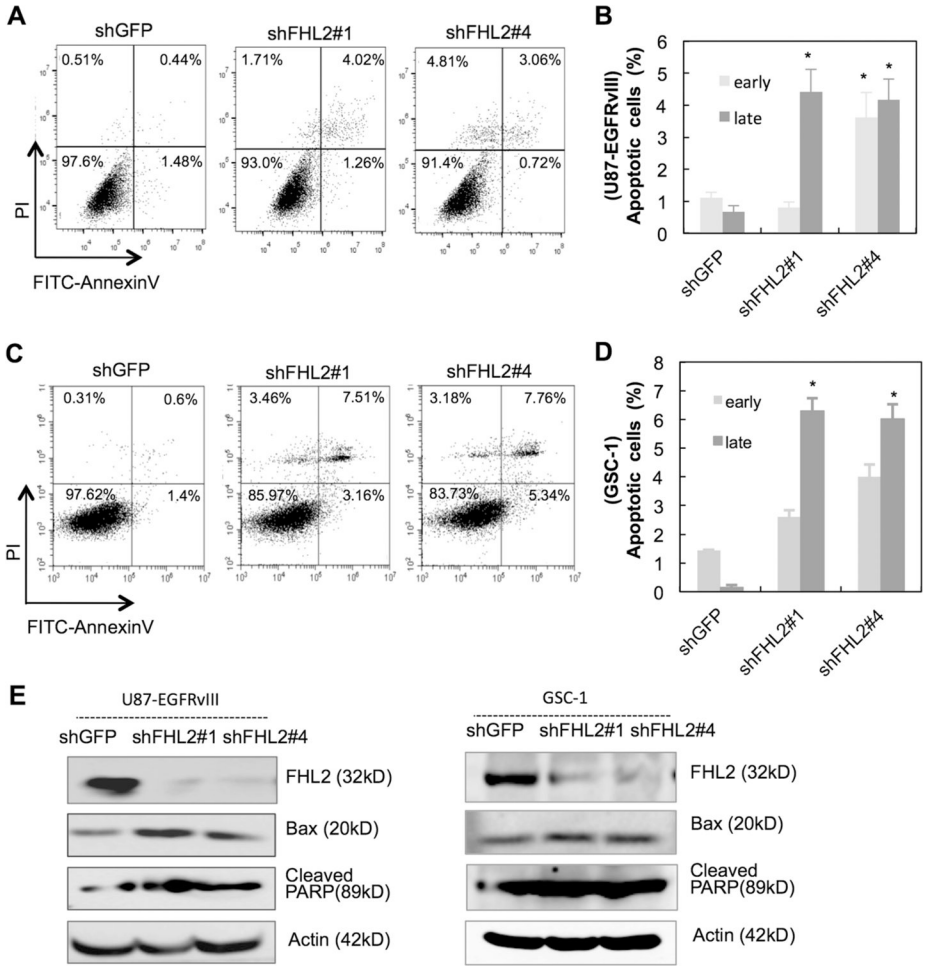

Fig. 5.

Targeting FHL2 induces cell apoptosis in EGFRvIII expressing GBM cells. **a**, **c** Two independent shRNA sequences (shFHL2#1 and shFHL2#4) or control shRNA (shGFP) were delivered to U87-EGFR-vIII and GSC-1 cells, respectively. The cells were serum starved for 24 h prior to PI/FITC-Annexin V staining. The apoptotic cells were detected with flow cytometry. The lower left panel shows the normal cells, the lower right panel shows the early apoptotic cells, and the upper right panel shows the late apoptotic cells or cells undergoing necrosis. Shown are representative of three independent experiments. **b, d** Quantitation of the apoptosis induced by FHL2 silencing in U87-EGFRvIII cells and GSC-1 cells of three independent experiments. $*p < 0.05$. **e** The expression of cell apoptosis markers in U87-EGFRvIII and GSC-1 cells with FHL2 silencing was analyzed with immunoblot. The data shown are representative of three independent experiments

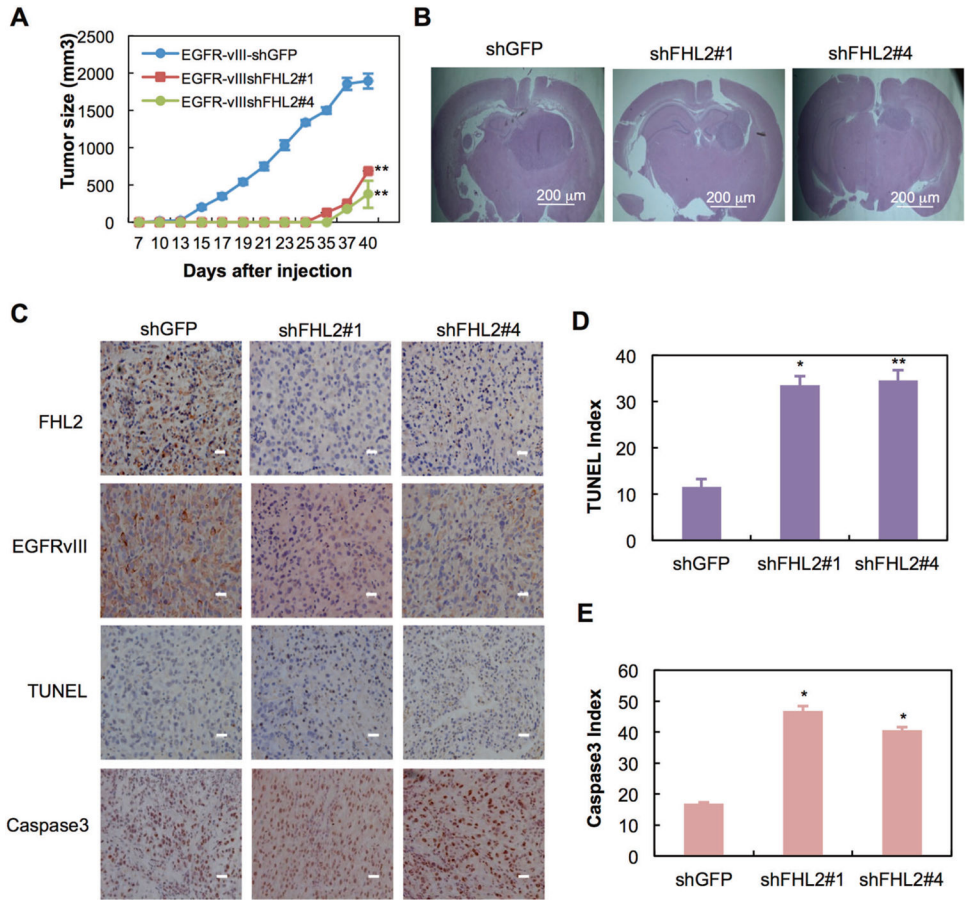

Fig. 6.

FHL2 is required for EGFRvIII-mediated tumor growth in vivo. **a** U87-EGFRvIII-shGFP, U87-EGFRvIII-shFHL2#1, and U87-EGFRvIII-shFHL2#4 flank xenograft tumor volume (mm^3) were measured at the indicated time in nude mice. 1×10^6 cells/mouse, 8 mice/group. $^{**}p < 0.01$. **b** H&E staining of brain sections on day 30 after intracranial inoculation of U87-EGFRvIII-shGFP (left), U87-EGFRvIII-shFHL2#1 (middle) or U87-EGFRvIII-shFHL2#4 (right). 2×10^5 cells/mouse, 6 mice/group. Shown are representative brain slices from tumor-bearing mice. Bars, 200 μm. **c** The expression levels of FHL2, EGFRvIII, and cell apoptosis markers (Caspase-3 and TUNEL) were analyzed with IHC staining. Bars, 20 μm. **d, e** The quantitative results of the TUNEL and Caspase-3 positive cells in U87-EGFRvIII-shGFP, U87-EGFRvIII-shFHL2#1, and U87-EGFR-vIII-shFHL2#4 tumors are shown. $^{*}p < 0.05$; $^{**}p < 0.01$