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Research Article

Oncology

Two hallmarks of glioblastoma multiforme, the most common malignant brain cancer in humans, are aggressive growth and the ability of single glioma cells to disperse throughout the brain. These characteristics render tumors resistant to current therapies and account for the poor prognosis of patients. Although it is known that oncogenic signaling caused by overexpression of genes such as *PDGFRA* is responsible for robust glioma growth and cell infiltration, the mechanisms underlying glioblastoma malignancy remain largely elusive. Here, we report that PDGFR α signaling in glioblastomas leads to Src-dependent phosphorylation of the guanine nucleotide exchange factor Dock180 at tyrosine 1811 (Dock180^{Y1811}) that results in activation of the GTPase Rac1 and subsequent cell growth and invasion. In human glioma cells, knockdown of Dock180 and reversion with an RNAi-resistant Dock180^{Y1811F} abrogated, whereas an RNAi-resistant Dock180^{WT} rescued, PDGFR α -promoted glioma growth, survival, and invasion. Phosphorylation of Dock180^{Y1811} enhanced its association with CrkII and p130^{Cas}, causing activation of Rac1 and consequent cell motility. Dock180 also associated with PDGFR α to promote cell migration. Finally, phosphorylated Dock180^{Y1811} was detected in clinical samples of gliomas and various types of human cancers, and coexpression of phosphorylated Dock180^{Y1811}, phosphorylated Src^{Y418}, and PDGFR α was predictive of extremely poor prognosis of patients with gliomas. Taken together, our findings provide insight into PDGFR α -stimulated gliomagenesis and suggest that phosphorylated Dock180^{Y1811} contributes to activation of Rac1 in [...]

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Activation of Rac1 by Src-dependent phosphorylation of Dock180^{Y1811} mediates PDGFR α -stimulated glioma tumorigenesis in mice and humans

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Two hallmarks of glioblastoma multiforme, the most common malignant brain cancer in humans, are aggressive growth and the ability of single glioma cells to disperse throughout the brain. These characteristics render tumors resistant to current therapies and account for the poor prognosis of patients. Although it is known that oncogenic signaling caused by overexpression of genes such as PDGFRA is responsible for robust glioma growth and cell infiltration, the mechanisms underlying glioblastoma malignancy remain largely elusive. Here, we report that PDGFR α signaling in glioblastomas leads to Src-dependent phosphorylation of the guanine nucleotide exchange factor Dock180 at tyrosine 1811 (Dock180^{Y1811}) that results in activation of the GTPase Rac1 and subsequent cell growth and invasion. In human glioma cells, knockdown of Dock180 and reversion with an RNAi-resistant Dock180^{Y1811F} abrogated, whereas an RNAi-resistant Dock180^{WT} rescued, PDGFR α -promoted glioma growth, survival, and invasion. Phosphorylation of Dock180^{Y1811} enhanced its association with CrkII and p130^{Cas}, causing activation of Rac1 and consequent cell motility. Dock180 also associated with PDGFR α to promote cell migration. Finally, phosphorylated Dock180^{Y1811} was detected in clinical samples of gliomas and various types of human cancers, and coexpression of phosphorylated Dock180^{Y1811}, phosphorylated Src^{Y418}, and PDGFR α was predictive of extremely poor prognosis of patients with gliomas. Taken together, our findings provide insight into PDGFR α -stimulated gliomagenesis and suggest that phosphorylated Dock180^{Y1811} contributes to activation of Rac1 in human cancers with PDGFRA amplification.

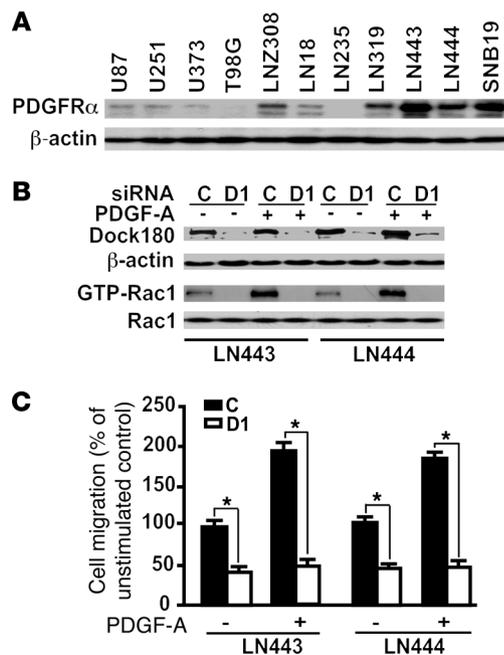
Introduction

Glioblastoma multiforme (GBM), the most common malignant brain cancer in humans, is characterized by high proliferation rates, extensive single-cell infiltration into the adjacent and distant brain parenchyma, and robust neoangiogenesis, which together inevitably confer resistance to current treatment modalities (1–3). Recently, coordinated genomic analyses of large cohorts of clinical GBM specimens rank PDGFR α third among the top 11 amplified genes in GBMs (4, 5). Further integrated analysis revealed that PDGFR α is preferentially amplified within a clinically relevant subtype of glioblastomas (6). Overexpression of PDGFR α and its ligand, PDGF-A, in clinical gliomas is associated with a poor prognosis and shorter survival time for patients (1–3). PDGFR α signaling promotes cell proliferation, survival, and motility through the PI3K, Src, and PLC γ pathways (7). Recently, we reported that activation of PDGFR α signaling drives gliomagenesis of *Ink4a/Arf*-deficient mouse astrocytes and human glioma cells in the brain (8).

Rac1 is a small Rho GTPase and a molecular switch essential for controlling cell movement, survival, and other cellular functions (9). Rac1 is activated by guanine nucleotide exchange factors (GEFs) that promote the exchange of GDP to GTP. There are 2 distinct families of Rho GEFs, those that contain a Dbl-homology (DH) domain and those that are devoid of it. The dedicator of cytokinesis (Dock) family of GEFs, with 11 members in humans, lacks DH domains, but instead has Dock-homology region-1 (DHR-1) and DHR-2 domains (10). Dock1 orthologs in *C. elegans*, *Drosophila*, and mammals (in which it is known as Dock180) modulate cell migration, myoblast fusion, dorsal closure, and cytoskeletal organization through activation of Rac1 (11). Dock180 facilitates GDP/GTP exchange of Rac1 through its DHR-2 domain, but requires formation of a complex with engulfment and cell motility 1 (ELMO1). This bipartite GEF complex synergistically functions upstream of Rac1 and promotes Rac1-dependent cell migration and phagocytosis (11). In cancers, Rac1 mediates tumor cell growth, survival, and invasion in response to various stimuli (9). Although neither Rac1 nor its GEFs, such as Dock180, are known to be overexpressed or mutated in human cancers (10), aberrant and constitutive activation of Rac1 might be involved in tumorigenesis and invasion.

Conflict of interest: The authors have declared that no conflict of interest exists.

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**Figure 1**

Dock180 mediates PDGFR α -stimulated glioma cell migration in vitro. (A) IB assays of endogenous PDGFR α expression in glioma cells. (B) LN443 and LN444 glioma cells were transiently transfected with a pool of Dock180 siRNA (D1) or a control siRNA (C). Rac-GTP levels were determined by GTPase activation assays. (C) In vitro cell migration assays were performed with a Boyden chamber using cells generated from B under serum-starvation conditions. Data are normalized with cell proliferation index (not shown) and presented as percent unstimulated control cells. * $P < 0.05$. Data (\pm SD) represent 3 independent experiments with similar results.

glioma cells. Additionally, in agreement with a previous report (17), *PDGFRA* gene amplification was observed in primary human glioma GBM5 cells, but not in GBM6 cells, GBM14 cells, or other cell lines examined (Supplemental Figure 1; supplemental material available online with this article; doi:10.1172/JCI58559DS1). Heterogeneous levels of PDGFR α expression in various glioma cell lines reflect preferential amplification of *PDGFRA* in the clinically relevant proneural subtype of glioblastomas, but not in other subclasses (6). Moreover, LN443 and LN444 cells also expressed a group of genes that were statistically similar to a subgroup of signature genes in the proneural subtype of clinical GBMs, such as *SOX3*, *GABRA3*, *GALNT13*, *MAPT*, and *NRXN2* (Supplemental Table 1 and ref. 6).

To determine whether Dock180 mediates PDGFR α -stimulated glioma cell growth and migration, we assessed the effect of Dock180 inhibition on PDGFR α stimulation. Knockdown of endogenous Dock180 by a pool of siRNAs markedly impaired basal and PDGF-A-stimulated Rac1 activities and in vitro cell migration of LN443 and LN444 cells (Figure 1, B and C). These data suggest that Dock180 is critical for PDGF-A/PDGFR α -induced Rac1 activation and glioma cell migration.

To determine the function of Dock180 in PDGFR α -stimulated tumorigenesis in vivo, we stably knocked down Dock180 in LN444/PDGFR α gliomas using 2 shRNAs (shRNA1 and shRNA2). Overexpression of PDGF-A by LN444 cells that express endogenous PDGFR α (referred to herein as LN444/PDGFR α cells) promoted the expression of a subgroup of the proneural signature genes (Supplemental Table 1) and gliomagenesis in the brain (8). Knockdown of Dock180 in LN444/PDGFR α cells did not affect expression of exogenous PDGF-A, endogenous PDGFR α , Akt, and Erk1/2 proteins, but reduced the PDGFR α -stimulated phosphorylation of Akt and Erk1/2 compared with the control (Figure 2A). Knockdown of Dock180 by 2 separate shRNAs in LN444/PDGFR α cells had a minimal effect on the population doubling times of cells cultured in media containing 10% FBS, compared with that of LN444/GFP or control shRNA (shControl) cells (Figure 2B). However, knockdown of Dock180 markedly decreased PDGF-A/PDGFR α -stimulated cell survival (Figure 2C; apoptotic index at 48 hours with starvation) and migration (data not shown). These data suggest that depletion of Dock180 inhibits PDGFR α -stimulated Rac1/MAPK and Rac1/Akt signaling as well as glioma cell survival and migration in vitro.

Next, we separately implanted LN444/PDGFR α /shControl, LN444/PDGFR α /Dock180-shRNA1 (referred to herein as shDock180-1), and LN444/PDGFR α /Dock180-shRNA2 (shDock180-2) cells into the brains of mice. Compared with control LN444/GFP gliomas, overexpression of PDGF-A by LN444 gliomas significantly enhanced tumor growth, survival, and invasion (8), whereas knockdown of Dock180 markedly suppressed PDGFR α -stimulated gliomagenesis in the brains of mice (Figure

Rac1 GEF couples receptor tyrosine kinases (RTKs) to Rac1 (12). PVR, a homolog of PDGF/VEGF receptor in *Drosophila*, is essential for cell migration and spatial guidance of embryonic blood cell precursors (13). Significantly, the Dock180/ELMO1 complex mediates PVR-induced cell migration of these precursor cells through Rac1 during *Drosophila* development (14). Previously, some of us reported that Dock180 plays a critical role in promoting glioma cell invasion through activation of Rac1 (15). Here, in order to determine whether Dock180 activation of Rac1 mediates PDGFR α signaling in glioblastomas, we examined the role of regulatory tyrosine phosphorylation (p-Y) of Dock180 in PDGFR α -promoted glioma tumorigenesis. Our results showed that Dock180 was specifically phosphorylated at tyrosine residue 1811 (p-Dock180^{Y1811}) by PDGFR α -activated Src kinase in gliomas, resulting in stimulation of Dock180 interaction with CrkII and p130^{Cas} as well as subsequent Rac1 activation that culminated in PDGFR α -promoted glioma growth, survival, and invasion. These findings suggest what we believe to be a previously unidentified intervention approach in the treatment of gliomas: targeting the PDGFR/Src/Dock180/Rac1 signaling cascade.

Results

Dock180 mediates PDGFR α -stimulated glioma cell migration and survival in vitro and tumor growth, survival, and invasion in the brain. To establish the role of Dock180 in PDGFR α -stimulated glioma growth, survival, and invasion, we first examined the expression of endogenous PDGFR α in various human glioma cell lines. The LNZ308, LN319, LN443, LN444, and SNB19 glioma cell lines endogenously expressed PDGFR α at moderate to high levels, whereas U87, U251, U373, T98G, LN18, and LN235 glioma cells had lower-level expression of PDGFR α (Figure 1A). To correlate expression levels of PDGFR α proteins with *PDGFRA* gene status in these glioma cells, we performed quantitative PCR analyses (16). Consistent with the levels of PDGFR α protein, increased copy numbers of the *PDGFRA* gene were found in LNZ308, LN319, LN443, LN444, and SNB19

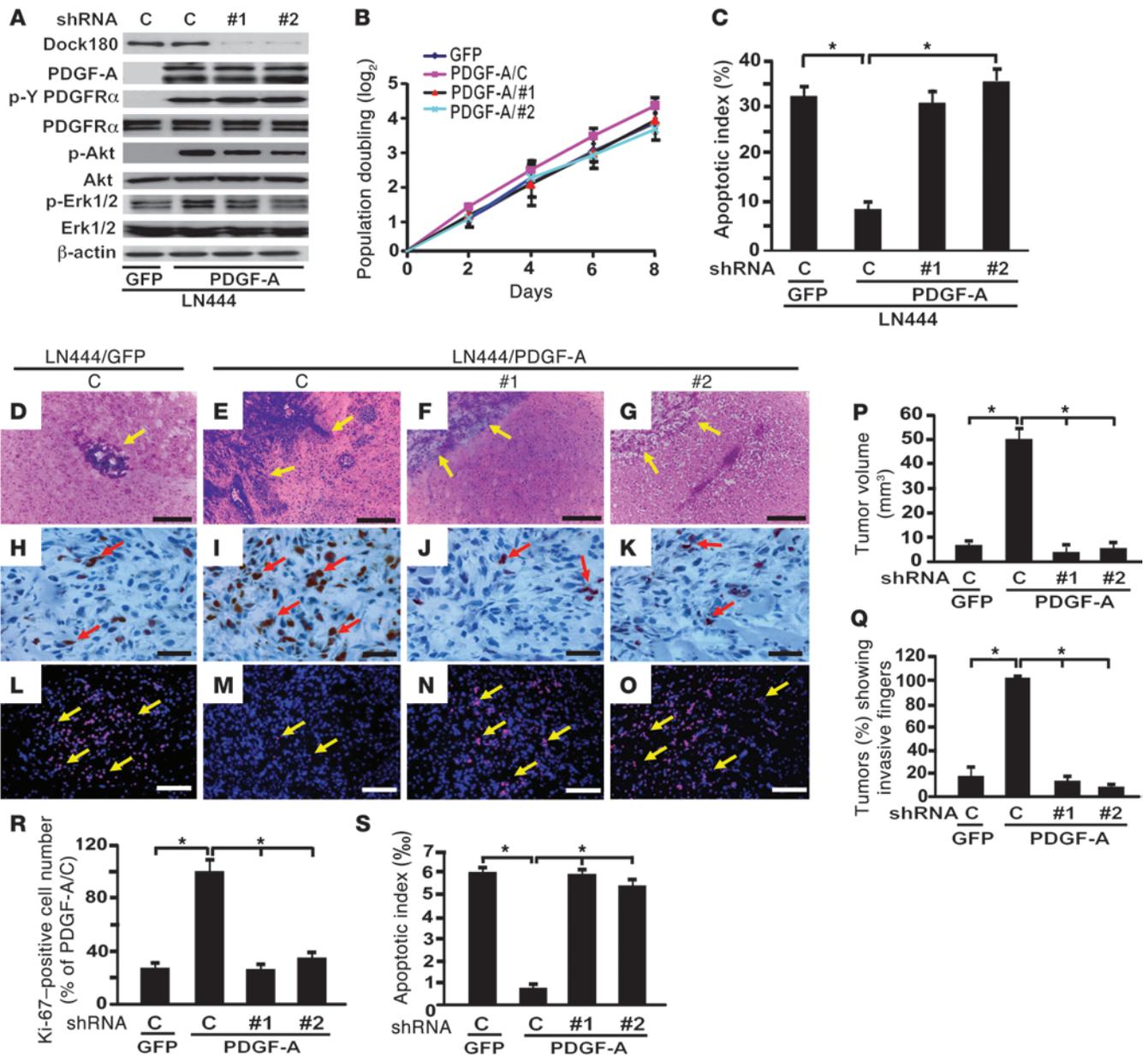


Figure 2

Dock180 mediates PDGFR α -stimulated glioma cell survival in vitro, tumor growth, and invasion in the brain. (A) Effect of knockdown of Dock180 with shRNA1 (#1), shRNA2 (#2), or shControl (C) on expression of PDGF-A, p-Y PDGFR α , p-AKT, and p-Erk1/2 in LN444 cells expressing PDGF-A or GFP. (B) Cell proliferation assays. (C) Cell viability, determined by TUNEL assays after 48-hour serum starvation. (D–O) Dock180 depletion inhibited PDGFR α -promoted LN444 glioma growth and invasion in the brain. Shown is representative H&E (D–G), Ki-67 (H–K), and TUNEL (L–O) staining of brain sections from 5 mice per group. Arrows denote invasive tumor cells (E), noninvasive tumor borders (D, F, and G), and positively stained tumor cells (H–O). Scale bars: 200 μ m (D–G); 50 μ m (H–K); 100 μ m (L–O). (P–S) Quantification of tumor size (P), percent of tumors showing invasive fingers (Q), Ki-67 staining (R), and TUNEL staining (S). Data (\pm SD) represent 3 independent experiments with similar results. * P < 0.05.

2, D–S, and Supplemental Figure 2). When various brain sections from the different tumors were examined by immunohistochemical (IHC) staining, ectopically expressed PDGF-A enhanced cell proliferation compared with the control (Figure 2, H, I, and R, and Supplemental Figure 2, I and J). However, depletion of Dock180 significantly decreased LN444 tumor cell proliferation compared with shControl tumors (Figure 2, I–K and R, and Supplemental

Figure 2, J–L). To a similar extent, compared with LN444/GFP, overexpression of PDGF-A resulted in a marked decrease in cell apoptosis (Figure 2, L, M, and S, and Supplemental Figure 2, N and M). Knockdown of Dock180 in LN444/PDGF-A tumors also significantly increased cell apoptosis compared with shControl tumors (Figure 2, M–O and S, and Supplemental Figure 2, N–P). Taken together, these results suggest that specific activation of

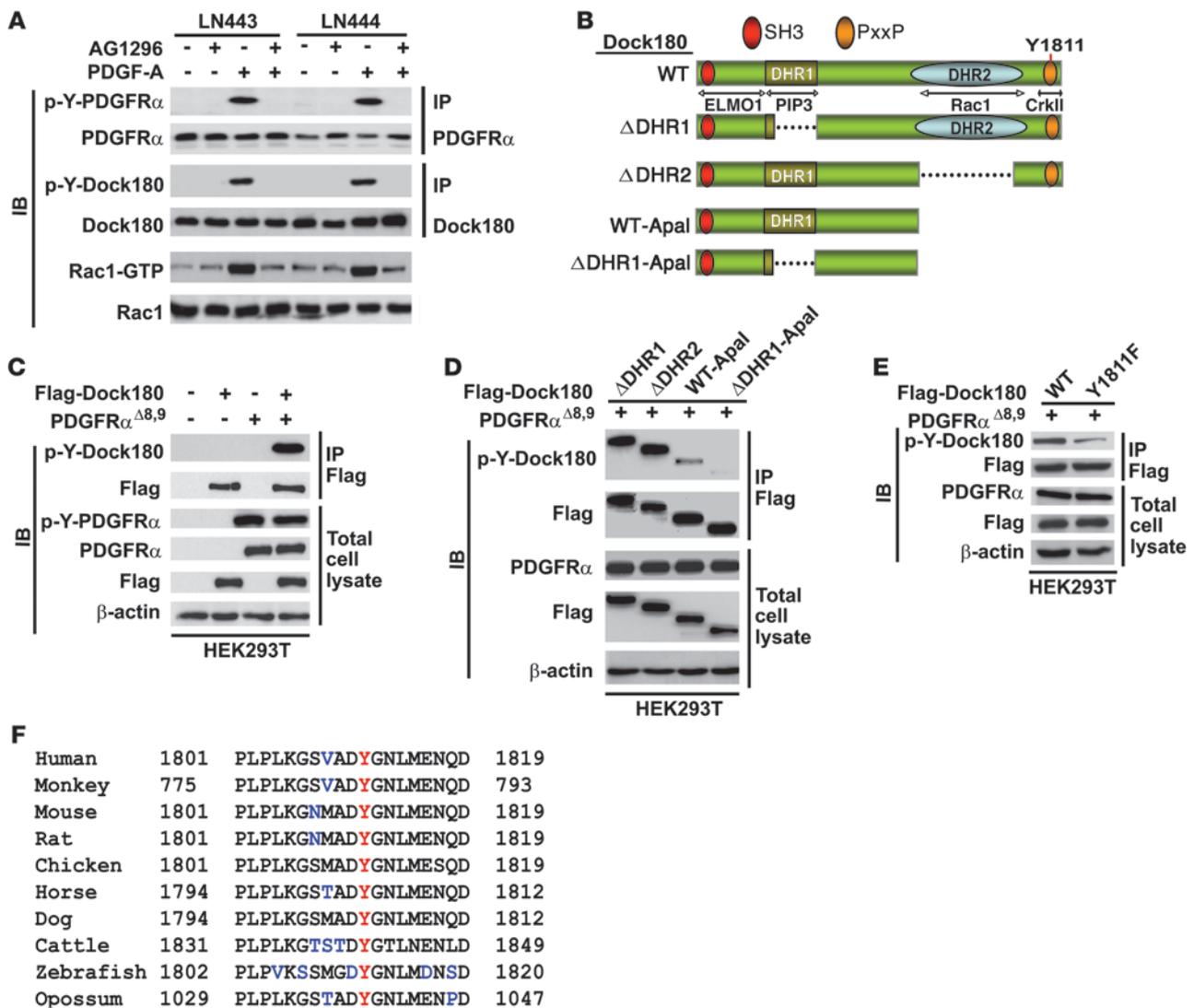


Figure 3

PDGF-A stimulation of PDGFR α induces p-Y of Dock180^{Y1811}. (A) PDGF-A induced p-Y of Dock180 and activated Rac1 in serum-starved LN443 and LN444 cells pretreated with or without the PDGFR α inhibitor AG1296 (20 μ M) for 1 hour followed by PDGF-A (50 ng/ml) treatment for 5 minutes. (B) Schematics of Dock180^{WT} and various Dock180 deletion mutants. (C and D) A CA PDGFR α (PDGFR $\alpha^{\Delta 8,9}$) induced p-Y of Dock180 at the Y1811 residue. Dock180^{WT} or the indicated Dock180 mutants and PDGFR $\alpha^{\Delta 8,9}$ were cotransfected into HEK293T cells. (E) Y1811 was a major PDGFR α -induced p-Y site. Experiments were similar to C, except Dock180^{WT} or Dock180^{Y1811F} was used. (F) Y1811 is conserved in Dock180 from various species. (A and C–E) p-Y of Dock180 was detected with a pan-phosphotyrosine antibody (4G10). Data represent 2 independent experiments with similar results.

PDGFR α by PDGF-A enhances glioma growth, survival, and invasion and that Dock180 is required for PDGFR α -promoted gliomagenesis in the brain.

Dock180 is specifically phosphorylated at Y1811 by PDGFR α stimulation in glioma cells. Since Dock180 activates Rac1 (11) and mediates PVR-induced cell migration in vivo (14), we hypothesized that PDGFR α signaling promotes glioma cell growth, survival, and invasion through p-Y of Dock180, leading to activation of Rac1. To test this hypothesis, we first examined whether PDGF-A stimulation induces p-Y of endogenous Dock180 in glioma cells. PDGF-A stimulation of LN443 or LN444 glioma cells induced distinct p-Y of PDGFR α , p-Y of Dock180, and activation of Rac1, whereas inhibition of PDGF-A stimulation by a PDGFR inhibitor, AG1296 (which selectively inhib-

its RTK activities of PDGFR α and PDGFR β and PDGF-mediated signaling in cells; ref. 18), impaired PDGF-A-induced p-Y of PDGFR α , p-Y of Dock180, and Rac1 activation in glioma cells (Figure 3A).

Next, we computationally examined potential p-Y sites of the Dock180 protein (using the SCANSITE tool, <http://scansite.mit.edu>; and the NetPhosK 1.0 server, <http://www.cbs.dtu.dk/services/NetPhosK>) and identified 27 hypothetical p-Y sites, including Y1811, a residue in Dock180 predicted to be a target of Src and other kinases. To determine whether PDGFR α stimulates glioma growth, survival, and invasion and Rac1 activation through one or more specific p-Y sites of Dock180, we constructed various Dock180 deletion mutants (Figure 3B) and cotransfected these DNAs, together with a glioma-derived and constitutively active (CA)

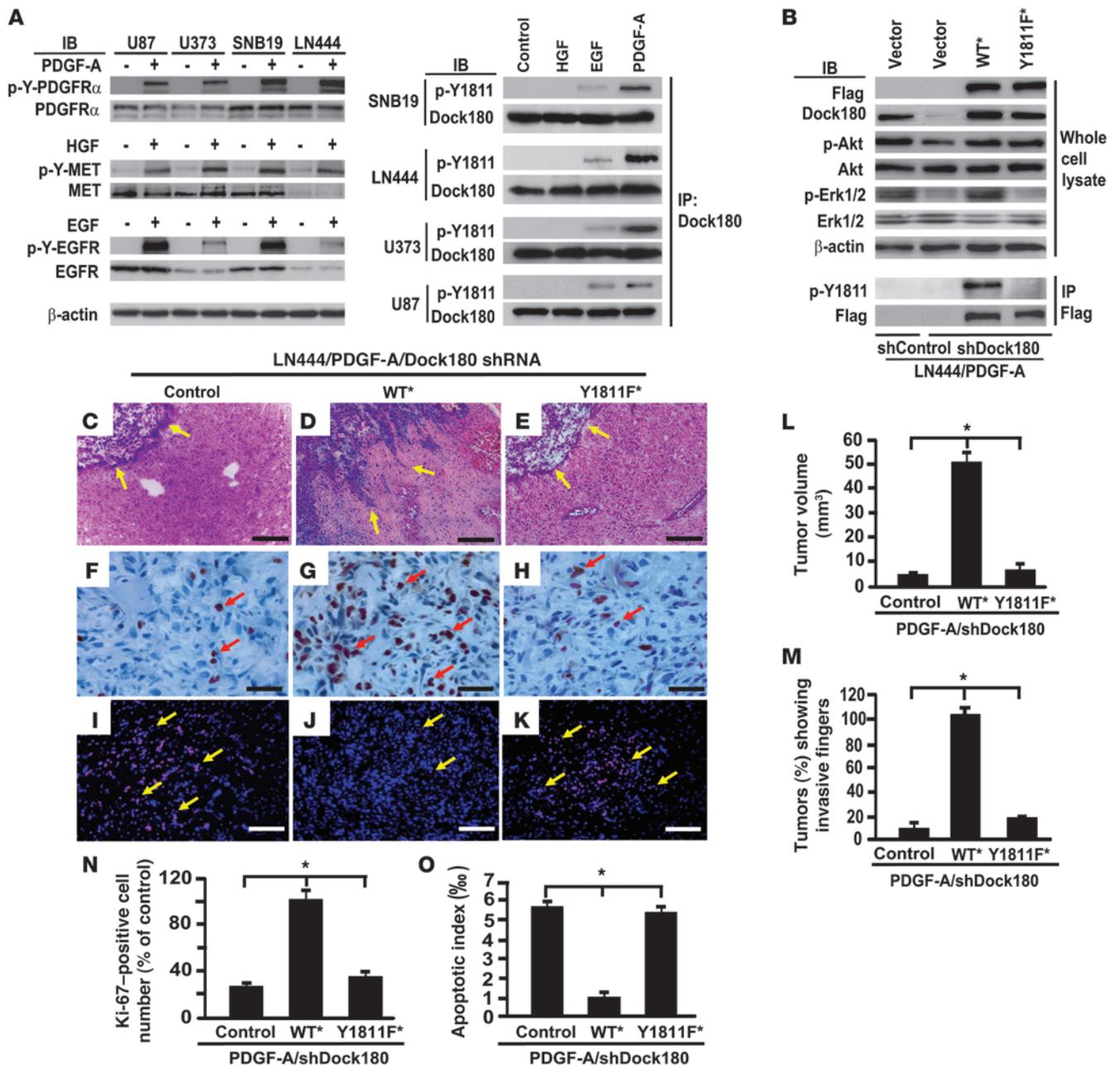


Figure 4

p-Y of Dock180^{Y1811} is required for PDGFR α -promoted glioma growth and invasion in the brain. (A) U87, U373, SNB19, and LN444 cells were serum-starved for 24 hours and treated with or without 50 ng/ml PDGF-A, 50 ng/ml EGF, or 40 ng/ml HGF for 5 minutes. Anti-c-Met (p-Y1230/Y1234/Y1235), anti-p-PDGFR α (p-Y754), and anti-p-EGFR (p-Y1045) antibodies were used to examine p-Y of c-Met, PDGFR α , and EGFR, respectively. (B) Effect of reexpression of shRNA-resistant Flag-tagged Dock180^{WT*}, Dock180^{Y1811F*}, or vector control on p-Akt and p-Erk1/2 in LN444/PDGFR-A/shRNA cells. (A and B) A specific anti-phosphotyrosine Dock180^{Y1811} antibody was used to detect p-Y of endogenous Dock180 in these cells. (C–K) Reexpression of shRNA-resistant Dock180^{WT*}, but not Dock180^{Y1811F*}, restored PDGFR α -promoted tumorigenesis of LN444/PDGFR-A/shRNA gliomas in the brain. Shown is representative H&E (C–E), Ki-67 (F–H), and TUNEL (I–K) staining of 5 mice per group. Arrows indicate invasive tumor cells (D), noninvasive tumor borders (C and E), or positively stained tumor cells (F–K). Scale bars: 200 μ m (C–E); 50 μ m (F–H); 100 μ m (I–K). (L–O) Quantifications of tumor size (L), percent of tumors showing invasive fingers (M), Ki-67 staining (N), and TUNEL staining (O). Data (\pm SD) represent 3 independent experiments with similar results. * P < 0.05.

PDGFR α ^{A8,9} mutant (19), into human HEK293T cells. Coexpression of PDGFR α ^{A8,9} with Dock180^{WT} induced p-Y of Dock180. Deletions of the DHR-1 (Δ DHR1) or DHR-2 (Δ DHR2) domain from the Dock180 peptide that interacts with PIP₃ or Rac1, respectively (11),

had no effect on PDGFR α ^{A8,9}-induced p-Y of Dock180 (Figure 3, C and D). However, removal of a C-terminal segment, including the DHR2 domain (WT-Apa1), markedly reduced PDGFR α ^{A8,9}-induced p-Y, whereas an N-terminal segment lacking the DHR-1 domain

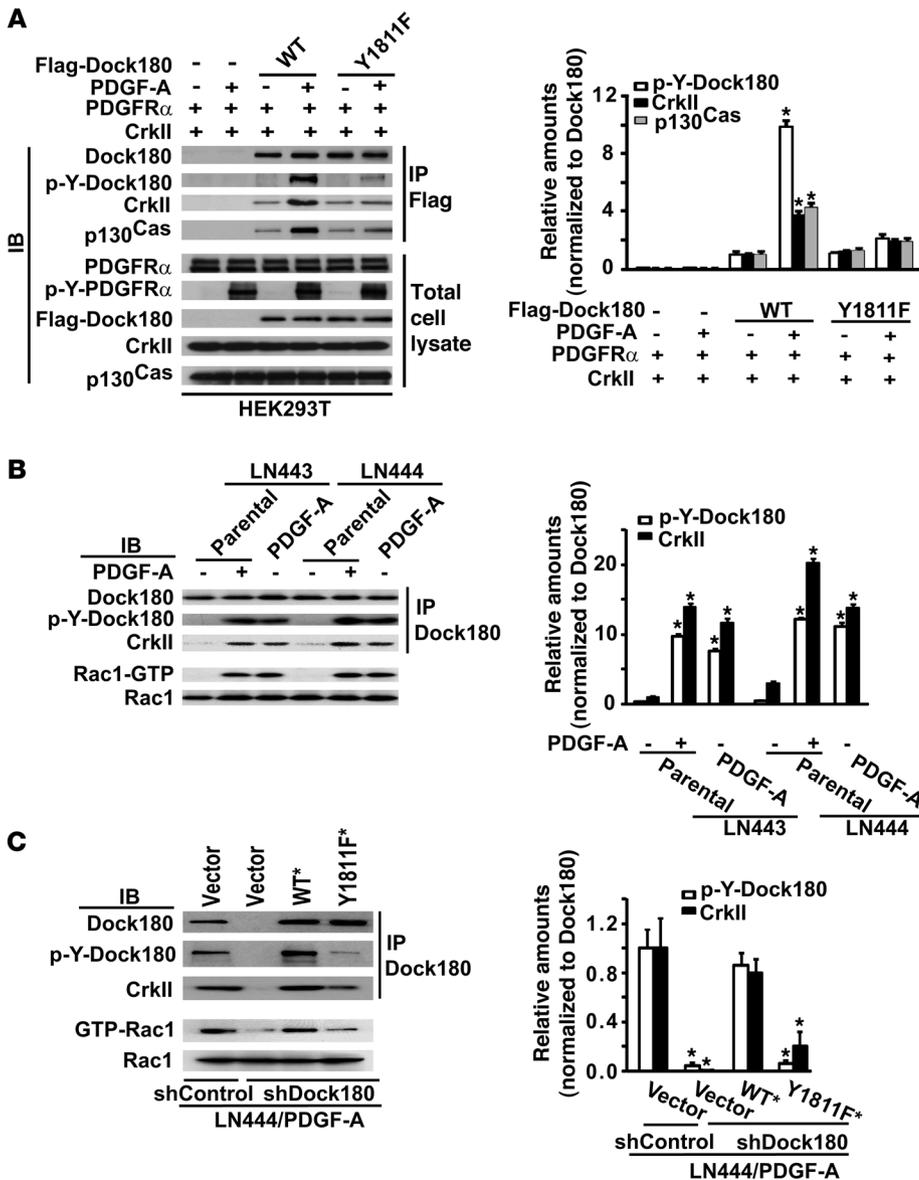


Figure 5 p-Y of Dock180^{Y1811} regulates PDGFR α -stimulated recruitment of CrkII and p130^{Cas} and activity of Rac1. (A) PDGF-A induced p-Y of PDGFR α and Dock180, but not Dock180^{Y1811F}, and protein association of Dock180^{WT}, but not Dock180^{Y1811F}, with CrkII and p130^{Cas}. (B) PDGF-A induced the formation of a Dock180/CrkII/p130^{Cas} complex and activated Rac1 in LN443 and LN444 cells stimulated by PDGF-A. (C) Reexpression of shRNA-resistant Flag-tagged Dock180^{WT*}, but not Dock180^{Y1811F*}, restored the PDGF-A-induced formation of a Dock180/CrkII/p130^{Cas} complex and activation of Rac1 in LN444/PDGF-A/shDock180 glioma cells. (A–C) p-Y of Dock180 was detected with a pan-phosphotyrosine antibody (4G10). Relative p-Y of Dock180, CrkII, or p130^{Cas} was determined by Image J and normalized to the amount of Dock180 in IP. **P* < 0.05. Data (\pm SD) represent 3 independent experiments with similar results.

(Δ DHR1-ApaI) further diminished the induced p-Y of Dock180, suggestive of a major p-Y site located at the C terminus. Examination of potential p-Y sites within the targeted domain of Dock180 identified a single p-Y candidate site at Y1811 (Figure 3B). To ascertain that Y1811 is a major p-Y site of Dock180 induced by PDGFR α , Y1811 was mutated to phenylalanine (F) in the full-length Dock180 protein. PDGFR α ^{A8,9}-induced p-Y was markedly reduced for Dock180^{Y1811F} compared with the stimulated p-Y of Dock180^{WT} (Figure 3E). Incomplete abrogation of PDGF-A-induced p-Y of Dock180 corroborates with the results in Figure 3D, which indicates that there is a minor p-Y site in the DHR-1 domain of Dock180 protein. When we examined aa sequences surrounding Y1811 in Dock180 of various species and Dock family members, we found that Y1811 and most of its surrounding aa residues were highly conserved in Dock180 among these species (Figure 3F), but not other members of the Dock family (Supplemental Figure 3). Taken together, these results suggest Y1811 of Dock180 as a major p-Y site that is specifically phosphorylated by PDGFR α in glioma cells.

p-Dock180^{Y1811} is required for PDGFR α -stimulated glioma cell migration and survival in vitro and tumor growth, survival, and invasion in the brain. We generated a rabbit polyclonal antibody that specifically recognizes the p-Dock180^{Y1811} protein. This anti-p-Dock180^{Y1811} antibody detected a strong signal of p-Y of endogenous Dock180 in U87, U373, SNB19, and LN444 glioma cells stimulated with PDGF-A, but weak or absent signal in these glioma cells treated with EGF or HGF, compared with the control (Figure 4A), indicative of its selectivity for PDGFR α -induced p-Dock180^{Y1811}. Next, we stably reexpressed an shRNA-resistant p-Dock180^{WT*} or Dock180^{Y1811F*} in poorly tumorigenic LN444/PDGF-A/shDock180-2 cells in which endogenous Dock180 had been stably depleted (Figure 2A). The exogenous Dock180^{WT*} or Dock180^{Y1811F*} proteins were expressed at levels comparable to those of endogenous Dock180 in shControl-expressing cells (Figure 4B). The anti-p-Dock180^{Y1811} antibody detected p-Y of Dock180^{WT*}, but not Dock180^{Y1811F*}, proteins in these cells. Moreover, Dock180^{WT*}, but not Dock180^{Y1811F*}, rescued the PDGF-A-induced phosphorylation of Erk1/2. However, both Dock180^{WT*} and Dock180^{Y1811F*}

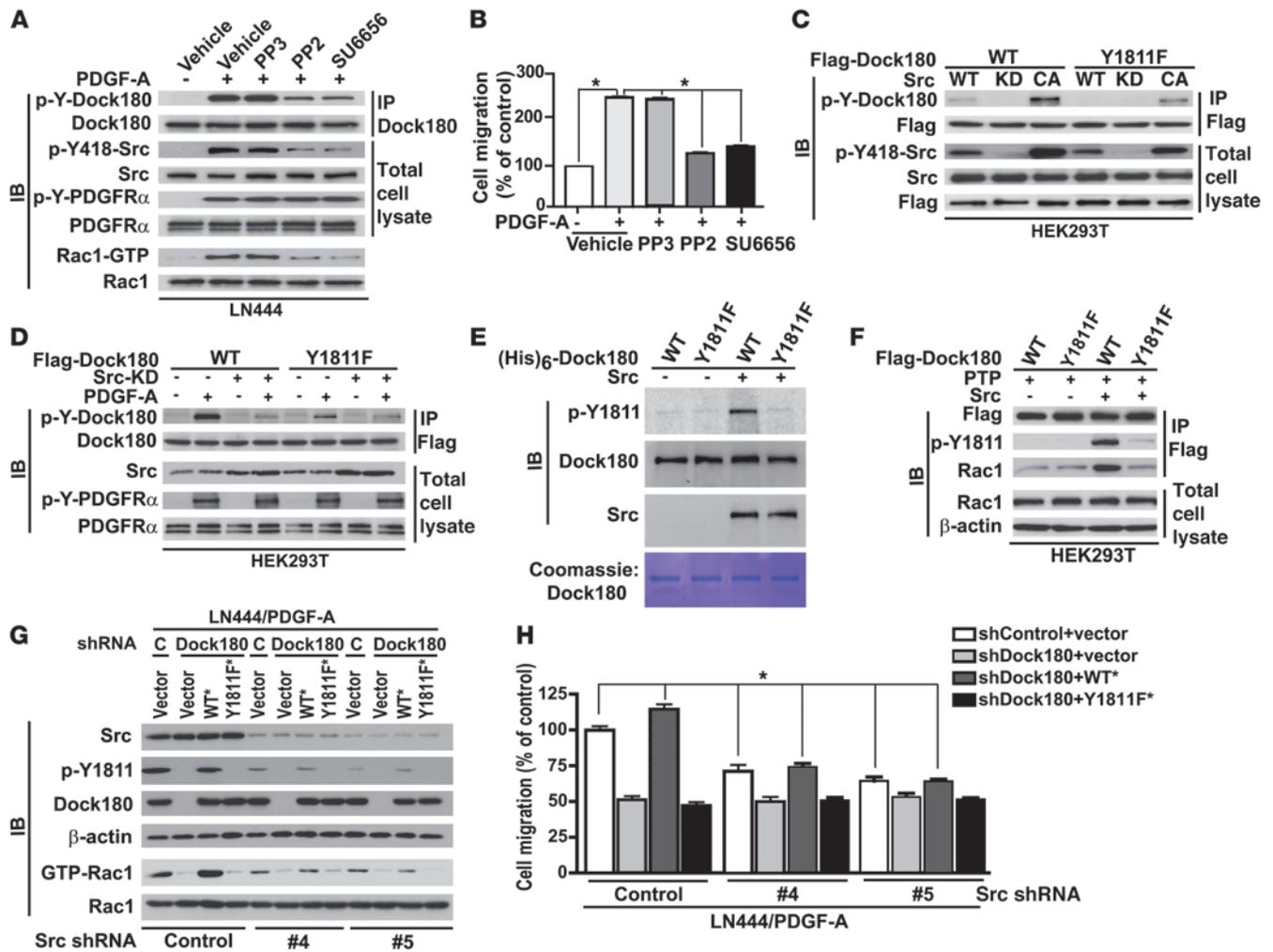


Figure 6

Src mediates PDGFR α stimulation of p-Y of Dock180^{Y1811}, activation of Rac1, and glioma cell migration. (A) Serum-starved LN444 cells were pretreated with or without PP3 (2 μ M), PP2 (2 μ M), and SU6656 (2 μ M) for 2 hours, and then stimulated with or without PDGF-A (50 ng/ml) for 5 minutes. (B) In vitro cell migration assays were performed and analyzed as in Figure 1C. (C) Src phosphorylates Dock180. Dock180^{WT} or Dock180^{Y1811F} and WT, KD, or CA Src were separately cotransfected into HEK293T cells. (D) Dock180^{WT} or Dock180^{Y1811F} and KD Src were separately cotransfected into HEK293T cells that were serum-starved and treated with or without PDGF-A. (E) In vitro Src kinase assay using recombinant active Src and (His)₆-Dock180^{WT} or (His)₆-Dock180^{Y1811F} proteins. Purified (His)₆-Dock180^{WT} or (His)₆-Dock180^{Y1811F} proteins were visualized by Coomassie brilliant blue staining. (F) Src phosphorylation of Dock180^{Y1811F} enhanced the association of Dock180 to Rac1. PTP, a recombinant YOP PTP. (G) Knockdown of Src inhibited PDGF-A–induced p-Y of Dock180^{Y1811} and Rac1 activation. LN444/PDGF-A/shDock180 or LN444/PDGF-A/shControl cells with or without restored Dock180^{WT*}, Dock180^{Y1811F*}, or pLVX-Puro vector control were transfected with Src shRNA4 (#4), Src shRNA5 (#5), or a shGFP control. (H) In vitro cell migration assays were performed and analyzed as in Figure 1C. (A, C, and D) p-Y of Dock180 was detected with a pan-phosphotyrosine antibody (4G10). (E–G) p-Y1811 was detected with a specific anti-p-Dock180^{Y1811} antibody. Data (\pm SD) represent 3 independent experiments with similar results. **P* < 0.05.

rescued the PDGF-A–induced phosphorylation of Akt (Figure 4B), which suggests that p-Dock180^{Y1811} is important for Rac1/MAPK signaling. Although no appreciable effect was seen on cell population doubling (Supplemental Figure 4A), reexpression of Dock180^{WT*}, but not Dock180^{Y1811F*}, in LN444/PDGF-A/shDock180-2 cells restored PDGF-A–stimulated cell viability and migration in vitro (Supplemental Figure 4, B and C). Importantly, when various engineered LN444 cells were implanted into the brains of mice, restoration of Dock180^{WT*}, but not Dock180^{Y1811F*}, in poorly tumorigenic LN444/PDGF-A/shDock180-2 cells rescued PDGFR α –promoted tumor growth, survival, and invasion (Figure 4, C–E, L, and M, and Supple-

mental Figure 5, A–F). Additionally, reexpression of Dock180^{WT*}, but not Dock180^{Y1811F*}, in LN444/PDGF-A/shDock180-2 gliomas also rescued PDGFR α –induced cell proliferation and inhibited cell apoptosis (Figure 4, F–K, N, and O, and Supplemental Figure 5, G–L). In addition, compared with the control or Dock180^{Y1811F*}, reexpression of Dock180^{WT*} by LN444/PDGF-A/shDock180-2 gliomas also significantly increased tumor angiogenesis and microglia growth, but had a minimal effect on the tumor infiltration of macrophages (Supplemental Figure 6). Taken together, these data suggest that p-Y of Dock180^{Y1811} is necessary for PDGFR α –stimulated glioma growth, survival, and invasion in the brain.

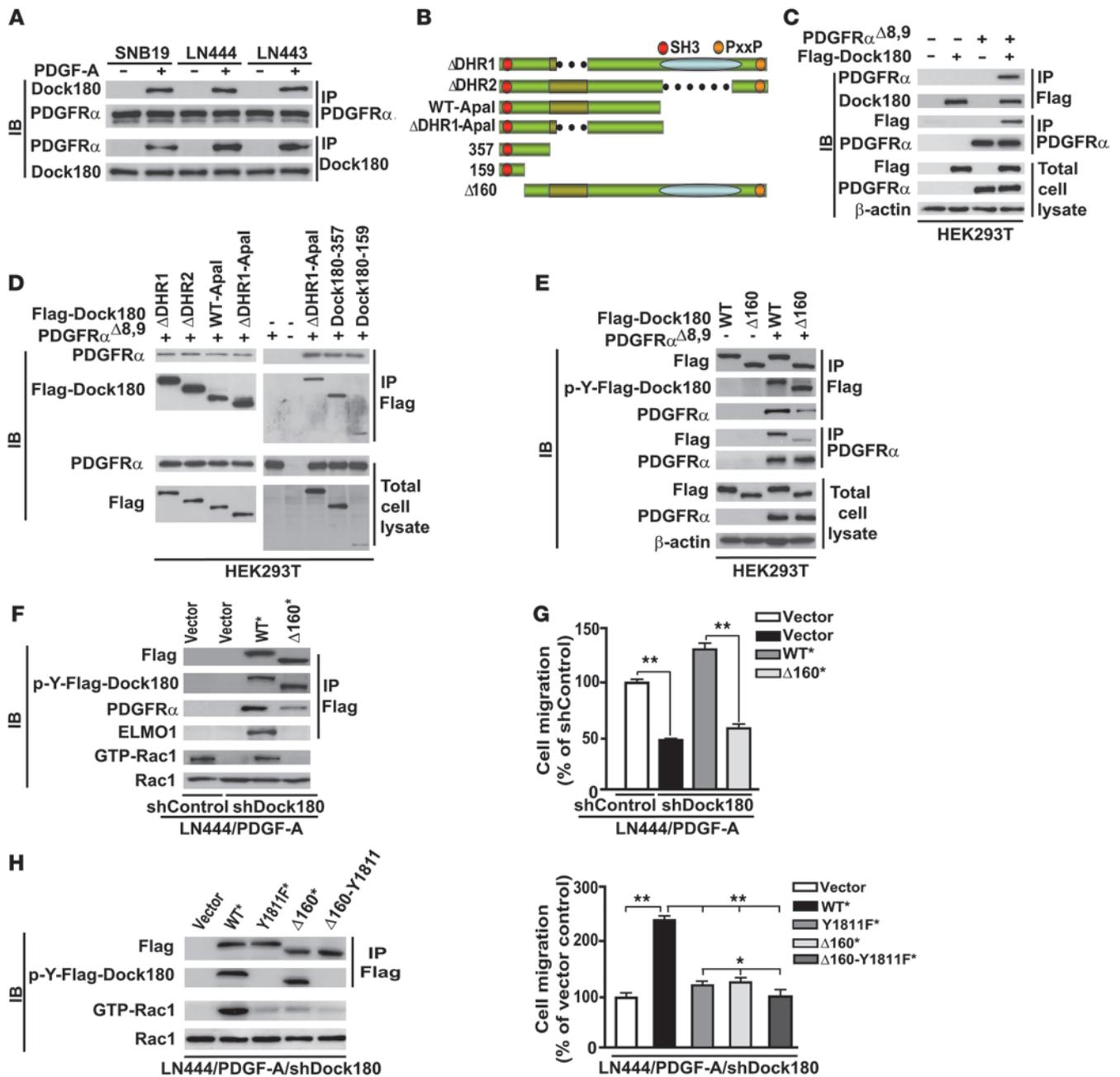


Figure 7 PDGF-A–induced association of Dock180 with PDGFR α is required for PDGFR α -promoted glioma cell migration. (A) PDGF-A induced association of Dock180 with PDGFR α in glioma cells. (B) Schematics of Dock180^{WT} and various Dock180 deletion mutants. (C–E) Dock180^{WT} or indicated Dock180 mutants was separately cotransfected with or without PDGFR $\alpha^{\Delta 8,9}$ into HEK293T cells. (C and D) Dock180 interacted with PDGFR α through its N-terminal region with aa residues 1–159. (E) Removal of 1–159 aa of Dock180 (Dock180 ^{Δ 160}) inhibited the induced interaction of Dock180 with PDGFR α without affecting p-Y Dock180 in HEK293T cells. (F) Dock180 ^{Δ 160} inhibited interaction of Dock180 with PDGFR α , ELMO1, and Rac1 activation without affecting p-Y of Dock180 in LN444/PDGF-A cells. (G) In vitro cell migration assays were performed and analyzed as in Figure 1C. (H) The double mutation Dock180 ^{Δ 160-Y1811F} had an additive effect on inhibition of PDGFR α -induced Rac1 activation and cell migration compared with that caused by single Dock180^{Y1811F} or Dock180 ^{Δ 160} mutation. (A, C–F, and H) Co-IP experiments were performed using anti-PDGFR α , anti-Dock180, anti-Flag, or anti-phosphotyrosine (4G10) antibodies. Data (\pm SD) represent 3 independent experiments with similar results. **P* < 0.05; ***P* < 0.001.

p-Dock180^{Y1811} mediates PDGFR α -stimulated recruitment of CrkII and p130^{Cas} and Rac1 activation. Y1811 is located at the C-terminal domain of Dock180, which interacts with CrkII and mediates Dock180/CrkII/p130^{Cas}/Rac1 signaling in promoting cell

motility (Figure 3B and ref. 11). Thus, we sought to determine whether p-Dock180^{Y1811} is critical for PDGFR α -induced formation of a Dock180/CrkII/p130^{Cas} complex and activation of Rac1 in glioma cells. WT PDGFR α and CrkII were coexpressed with

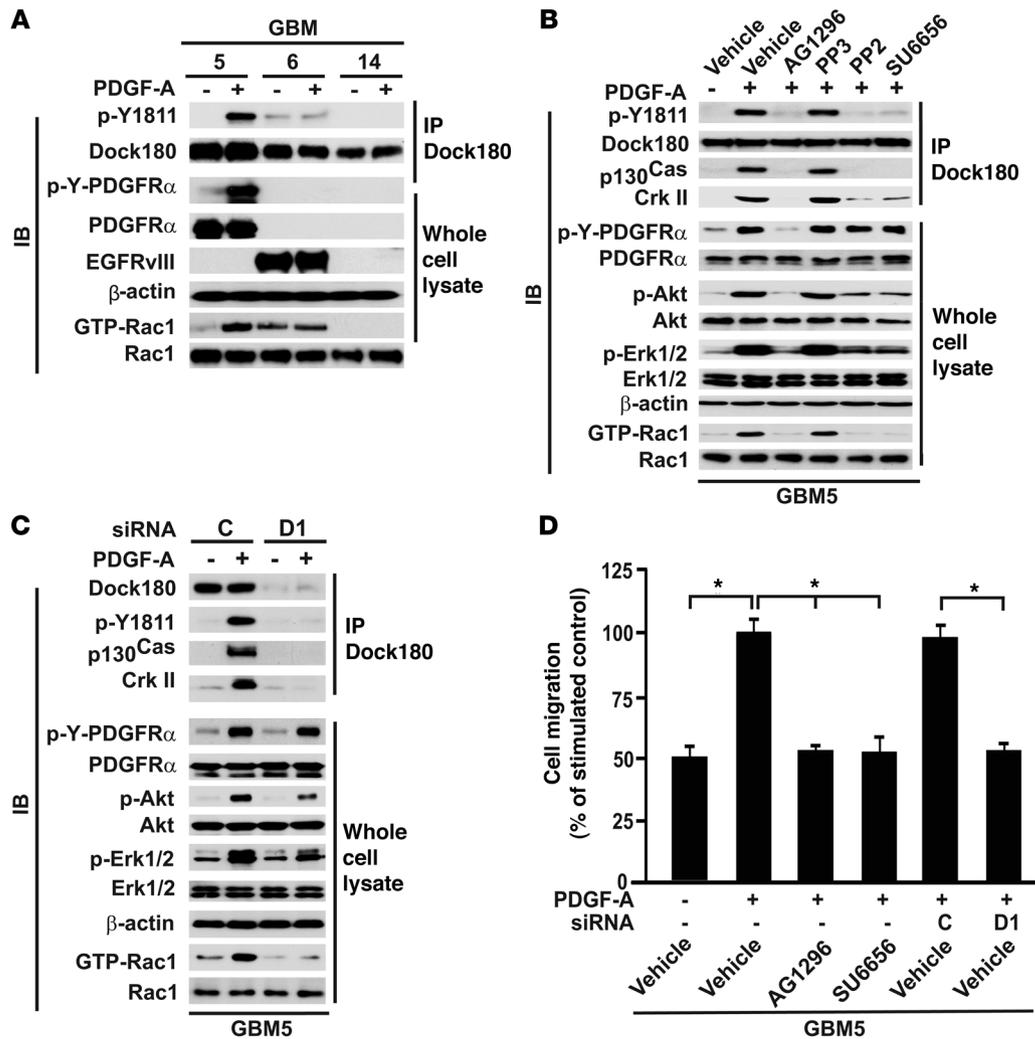


Figure 8

Inhibition of p-Dock180^{Y1811} by inhibitors of PDGFR α or Src, or by Dock180 siRNA, impairs PDGFR α -stimulated Dock180 association with p130^{Cas}, CrkII, p-Akt, p-Erk1/2, and Rac1 activities as well as cell migration in GBM5 cells with PDGFR α overexpression. (A) PDGF-A stimulation promoted p-Y of Dock180^{Y1811} in GBM5 cells. (B) PDGFR α or Src inhibitors blocked PDGFR α -stimulated p-Y of Dock180^{Y1811} as well as Dock180 association with p130^{Cas}, CrkII, p-Akt, p-Erk1/2, and Rac1 activities in GBM5 cells. (C) Knockdown of Dock180 by siRNA inhibited PDGFR α -stimulated p-Y of Dock180^{Y1811} and Dock180 association with p130^{Cas}, CrkII, p-Akt, p-Erk1/2 and Rac1 activities in GBM5 cells. C, control siRNA; D1, Dock180 siRNA pool. (D) Treatment with PDGFR α or Src inhibitors or Dock180 siRNA inhibited PDGFR α -stimulated cell migration in GBM5 cells. (A–C) An anti-p-Dock180^{Y1811} antibody was used to detect p-Dock180^{Y1811} (p-Y1811). Data (\pm SD) represent 3 independent experiments with similar results. * $P < 0.05$.

either Flag-tagged Dock180^{WT} or Flag-tagged Dock180^{Y1811F} in HEK293T cells. PDGF-A stimulation induced p-Y of WT PDGFR α and Dock180^{WT} and association of Dock180^{WT} with CrkII and p130^{Cas} (Figure 5A). In contrast, co-IP experiments showed that the Dock180^{Y1811F} mutation substantially attenuated PDGFR α -induced p-Y of Dock180 and interaction of Dock180 with CrkII and p130^{Cas}, whereas no effect on p-Y of PDGFR α was observed. Treatment of LN443 or LN444 cells with exogenous PDGF-A or ectopic PDGF-A expression induced the association of Dock180 with CrkII and p130^{Cas} and activated Rac1 (Figure 5B). However, restoration of Dock180^{Y1811F*}, but not Dock180^{WT*}, in LN444/PDGF-A/shDock180 cells in which endogenous Dock180 had been depleted impeded PDGF-A induction of p-Dock180^{Y1811}, association of Dock180 with CrkII, and activation of Rac1

(Figure 5C). These results indicate that p-Dock180^{Y1811} mediates PDGFR α -induced association of Dock180 with CrkII and p130^{Cas} and activation of Rac1 in glioma cells.

Src mediates PDGFR α stimulation of p-Dock180^{Y1811} and glioma cell migration. In addition to the potential p-Y sites of Dock180, our computational analysis also suggested Dock180^{Y1811} as a putative substrate site for Src. Src is a non-RTK that plays a crucial role in tumor progression and tumorigenesis promoted by aberrant activation of RTK signaling, including PDGFR (20). Thus, we hypothesized that PDGFR α promotes glioma tumorigenesis and invasion through Src stimulation of p-Dock180^{Y1811} and activation of Rac1. To test this hypothesis, LN444 cells were pre-treated with or without the Src inhibitor PP2, its PP3 inactive stereoisomer, or SU6656, a structurally unrelated Src inhibitor,

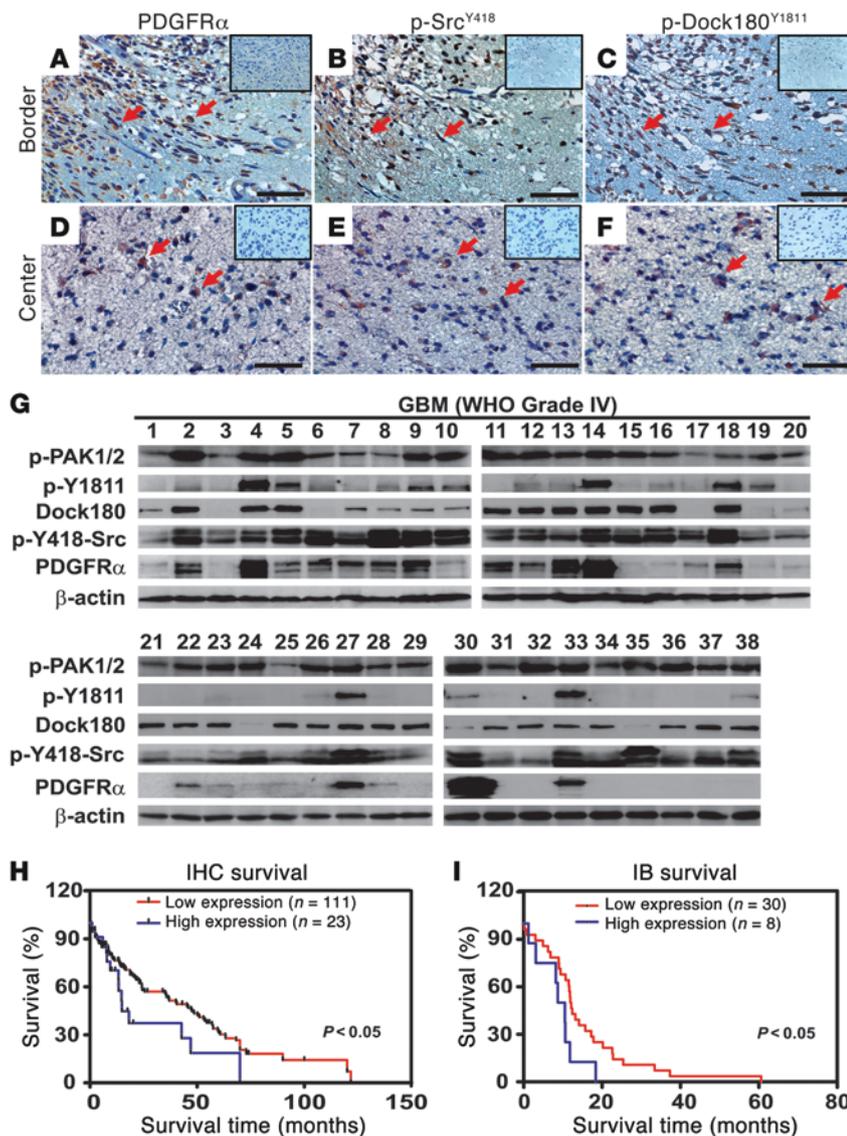


Figure 9 p-Dock180^{Y1811}, p-Src^{Y418}, and PDGFR α are coexpressed in primary human glioma specimens. (A–F) In total, 134 clinical primary glioma specimens, including WHO grades II–IV, were analyzed by IHC staining for PDGFR α , p-Src^{Y418}, and p-Dock180^{Y1811}. Specimens expressing PDGFR α and/or p-Dock180^{Y1811} are listed in Supplemental Table 2. Representative images of serial sections of a WHO grade IV GBM tissue using anti-PDGFR α (A and D), anti-p-Src^{Y418} (B and E), and anti-p-Dock180^{Y1811} (C and F) antibodies are shown. (A–C) Invasive border area. (D–F) Central region. Insets show isotype-matched IgG controls of the identical area (original magnification, $\times 400$). Arrows denote positive staining. Scale bars: 50 μ m. (G) Expression of PDGFR α , p-Src^{Y418}, p-Dock180^{Y1811} (using the specific anti-p-Dock180^{Y1811} antibody), and p-PAK1/2 (p-PAK1^{T423}/PAK2^{T402}) in a separate and independent cohort of a total of 38 snap-frozen GBM specimens. Dock180 and β -actin were used as loading controls. (H and I) Kaplan-Meier analyses of patients with high PDGFR α /p-Dock180^{Y1811}-expressing tumors versus low PDGFR α /p-Dock180^{Y1811}-expressing tumors in IHC staining (H) and IB (I) assays of WHO grade II–IV gliomas. *P* values were calculated by log-rank test. Black bars, censored data. Data represent 2 independent experiments with similar results.

prior to stimulation with PDGF-A. Treatment of LN444 cells with PP2 or SU6656, but not vehicle control or PP3, markedly decreased PDGF-A-induced p-Y of Dock180, p-Src^{Y418}, and Rac1 activity (Figure 6A). Of note, the PDGF-A-induced p-Y of PDGFR α remained unchanged in the presence or absence of the Src inhibitors. When various treated LN444 cells were analyzed for in vitro migration, inhibition of Src by PP2 or SU6656, but not the control or PP3, significantly abrogated PDGF-A-induced cell migration (Figure 6B). The incomplete inhibition of PDGF-A-induced p-Y of Dock180 by Src inhibitors could be due to a minor p-Y site in Dock180 (Figure 3), which suggests that other kinases, such as PI3K, may contribute to p-Y of Dock180. To test this possibility, we treated LN444 cells with the PI3K inhibitor LY294002 prior to PDGF-A stimulation. We found that inhibition of PI3K did not affect PDGF-A-induced p-Y of Dock180, but markedly decreased PDGF-A-induced Rac1 activity and cell migration, compared with controls (Supplemental Figure 7 and ref. 21), which suggests that a different kinase mediates PDGF-A-induced p-Y at this minor site.

To determine whether Src phosphorylates Dock180^{Y1811}, we separately cotransfected cDNAs of Flag-tagged Dock180^{WT} or Flag-tagged Dock180^{Y1811F} together with Src WT, a kinase-dead (KD) mutant, or a CA mutant (Y527F) into HEK293T cells. Without stimulation, CA Src, but not WT or KD Src, induced p-Y of Flag-tagged Dock180^{WT} (Figure 6C). In contrast, coexpression of Flag-tagged Dock180^{Y1811F} with CA Src severely reduced its p-Y, whereas no p-Dock180^{Y1811F} was detected when WT or KD Src were coexpressed in HEK293T cells. To validate that Src mediates PDGFR α stimulation of p-Dock180^{Y1811}, Flag-tagged Dock180^{WT} and Flag-tagged Dock180^{Y1811F} were separately cotransfected with WT PDGFR α and KD Src mutant into HEK293T cells. After 48 hours, cells were serum starved and stimulated with PDGF-A. Treatment with PDGF-A induced p-Y of PDGFR α and Dock180, whereas coexpression of KD Src markedly inhibited PDGF-A-induced p-Y of Dock180, with no effect on PDGF-A-induced p-Y of PDGFR α (Figure 6D). Furthermore, Dock180p^{Y1811F} significantly prevented PDGF-A-stimulated p-Y of Dock180, but not p-Y of PDGFR α . Coexpression of KD Src with Dock180^{Y1811F} further diminished

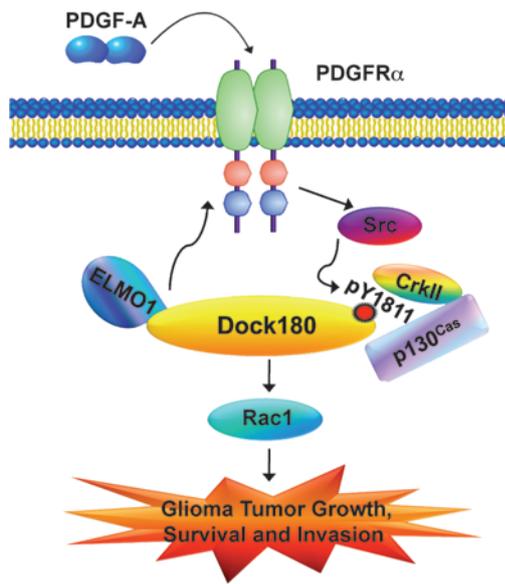


Figure 10
Working model of PDGFR α /Src/Dock180/CrkII/p130^{Cas}/Rac1 signaling in glioma tumor growth, survival, and invasion. PDGF-A activation of PDGFR α induces Src-dependent p-Y of Dock180^{Y1811} at the C terminus, promoting its association with CrkII and p130^{Cas} as well as Rac1 activities and leading to increased glioma tumor growth, survival, and invasion. PDGF-A activation of PDGFR α also induces an association of the receptor with the N terminus of Dock180, resulting in enhanced Rac1 activities and glioma migration.

PDGF-A-stimulated p-Y of Dock180 (Figure 6D). Again, similar to our data in Figure 3, the residual PDGF-A-induced p-Y of Dock180 (Figure 6, C and D) was attributable to the existence of a minor p-Y site in Dock180 that is a substrate for another kinase.

Next, we performed an *in vitro* p-Y assay by incubating purified recombinant (His)₆-Dock180^{WT} or (His)₆-Dock180^{Y1811F} proteins with a recombinant active Src. Dock180^{WT}, but not Dock180^{Y1811F}, was phosphorylated by Src (Figure 6E). Next, we performed a reconstitution assay for nucleotide-free Rac binding (22, 23). In the absence of the recombinant Src, when immunoprecipitated Dock180^{WT} or Dock180^{Y1811F} was dephosphorylated by a protein tyrosine phosphatase (PTP), minimal Dock180-Rac1 interaction was seen. However, when a recombinant Src was added, substantial p-Dock180^{WT}, but not p-Dock180^{Y1811F}, was induced, accompanied with an increase in association of Dock180 with Rac1 (Figure 6F). Finally, we knocked down endogenous Src using 2 separate shRNAs (shRNA4 and shRNA5) in LN444/PDGFR α /shDock180 cells with or without reexpression of Dock180^{WT*} or Dock180^{Y1811F*} (Figure 6G). p-Dock180^{Y1811} and Rac1 activation were observed in LN444/PDGFR α /shControl or Dock180^{WT*}-reexpressing cells. However, effective depletion of Src markedly decreased PDGF-A-induced p-Y of Dock180^{WT*} and Rac1 activity in LN444/PDGFR α /shControl and Dock180^{WT*}-restored cells, but had no effect in vector or Dock180^{Y1811F*} cells. As a result, knock-down of Src also inhibited PDGF-A stimulation of cell migration in the control and Dock180^{WT*}-restored cells (Figure 6H). Taken together, these data indicate that Src mediates PDGFR α stimulation of glioma cell migration through specific p-Y of Dock180^{Y1811} and activation of Rac1.

PDGF-A-induced association of Dock180 with PDGFR α is necessary for cell migration. We sought to determine whether endogenous Dock180 also associates with PDGFR α in glioma cells by separately treating SNB19, LN444, and LN443 cells with PDGF-A. Treatment with PDGF-A induced an association of Dock180 with PDGFR α in all 3 cell lines (Figure 7A). To identify which region or domain in Dock180 mediates its association with PDGFR α , we generated several deletion mutants lacking various functional binding domains (Figure 3B and Figure 7B). When these Dock180 deletion mutants were separately cotransfected with PDGFR α ^{A8,9} into HEK293T cells, all Dock180 deletion mutants – as well as a short fragment of Dock180, 1–159 mutant – were able to interact with CA PDGFR α ^{A8,9} (Figure 7, C and D). This suggests that the N-terminal region of aa residues 1–159 of Dock180 protein is involved in its association with activated PDGFR α . To confirm this, we coexpressed PDGFR α ^{A8,9} with a Flag-tagged Dock180 deletion mutant that lacks its N-terminal 1–159 aa residues (Dock180^{A160}) in HEK293T cells and found that the induced Dock180 association with PDGFR α ^{A8,9} was abrogated in cells (Figure 7E), whereas the Dock180^{Y1811F} mutant did not affect Dock180 association with PDGFR α ^{A8,9} (Supplemental Figure 8). To further examine whether this association affects PDGF-A stimulation of p-Y of Dock180 and glioma cell migration, we used LN444/PDGFR α /shDock180-2 cells, in which PDGFR α was activated by PDGF-A expression and endogenous Dock180 was stably depleted (Figure 2A). When a shRNA-resistant Dock180^{WT*} or a shRNA-resistant Dock180^{A160*} mutant were separately reexpressed, Dock180^{WT*} restored PDGF-A-induced association of Dock180 and ELMO1 with WT PDGFR α and activation of Rac1, whereas Dock180^{A160*} markedly inhibited its association with PDGFR α and ELMO1 as well as Rac1 activation (Figure 7F). Furthermore, restoration of Dock180^{WT*} enhanced PDGF-A-stimulated LN444/PDGFR α /shDock180-2 cell migration, whereas reexpression of the Dock180^{A160*} mutant was unable to render the cells responsive to PDGF-A stimulation of cell migration (Figure 7G). Finally, reexpression of a shRNA-resistant Dock180^{A160-Y1811F*} mutant (which lacks both the N-terminal 1–159 aa residues and the Y1811 phosphorylation site) attenuated PDGF-A stimulation of Rac1 activities and had an additive effect on reducing cell migration compared with that caused by individual mutations (Figure 7H). Together, these results demonstrated that the N-terminal region of Dock180 (aa residues 1–159) formed a complex with PDGFR α and ELMO1 and modulated PDGF-A-stimulated glioma cell migration without affecting the induced p-Y of Dock180.

Src-dependent p-Dock180^{Y1811} is important for PDGF-A-induced Dock180 association with p130^{Cas} and CrkII; activation of Akt, Erk1/2, and Rac1; and cell migration of primary GBM cells with PDGFR α amplification. To determine whether Src-dependent p-Y of Dock180^{Y1811} is required for Rac1 activation and cell migration in primary GBM cells with PDGFR α overexpression, we used primary GBM5 (PDGFR α gene amplification), GBM6 (EGFR gene amplification and EGFRvIII overexpression), and GBM14 (no PDGFR α or EGFR gene amplification) cells (Supplemental Figure 1 and refs. 17, 24). PDGF-A stimulation of primary GBM5 cells with PDGFR α overexpression increased p-Y of Dock180^{Y1811} and Rac1 activities compared with GBM6 and GBM14 cells (Figure 8A). Consistent with our results in Figure 4A, GBM6 cells with EGFRvIII overexpression minimally increased p-Y of Dock180^{Y1811} and increased Rac1 activities with or without PDGF-A stimulation. PDGFR α stimulation also promoted the association among Dock180, p130^{Cas}, CrkII, p-Akt, p-Erk1/2, and Rac1 activities in GBM5 cells, whereas AG1296, PP2, and SU6656 attenuated PDGF-A-induced p-Y of Dock180^{Y1811} and



the association of Dock180, p130^{Cas}, CrkII, p-Akt, p-Erk1/2, Rac1 activities, and cell migration of GBM5 cells (Figure 8, B and D). To further define this signaling, we transiently transfected GBM5 cells with a Dock180 siRNA pool or control siRNA. Knockdown of Dock180 also inhibited PDGF-A stimulation of these biochemical and cellular behaviors in GBM5 cells (Figure 8, C and D). Therefore, these results recapitulated our *in vitro* and *in vivo* observations in LN443 and LN444 cells, which further suggests that Src-dependent p-Dock180^{Y1811} is critical for PDGFR α -stimulated Dock180 association with p130^{Cas}, CrkII, p-Akt, p-Erk1/2, Rac1 activities, and cell migration in glioma cells.

p-Dock180^{Y1811} is present with PDGFR α and p-Src^{Y418} in human clinical glioma specimens. Based on accumulating evidence supporting an important function of p-Dock180^{Y1811} in PDGFR α -stimulated glioma tumorigenesis, we sought clinical evidence for a link among p-Dock180^{Y1811}, PDGFR α , and active Src. We immunostained a total of 134 clinical glioma tumor samples using an anti-PDGFR α antibody. PDGFR α proteins were detected in 3 of 26 WHO grade II gliomas (11.5%), 6 of 30 WHO grade III gliomas (20%), and 24 of 78 GBM specimens (30.8%), similar to previously reported frequencies (4, 6). Subsequently, we performed IHC analyses of these PDGFR α -expressing GBM specimens using anti-p-Src^{Y418} and our specific anti-p-Dock180^{Y1811} antibodies. PDGFR α protein was detected in both invasive and central regions in the GBM tumors and in WHO grade II and III tumors (Figure 9, A–F, Supplemental Figures 9 and 10, and data not shown). Notably, both p-Src^{Y418} and p-Dock180^{Y1811} were also expressed in the majority of PDGFR α -positive tumor cells in invasive and central regions of clinical glioma specimens (Figure 9, B, C, E, and F, Supplemental Figure 10, B, C, E, and F, and Supplemental Table 2). In contrast, minimal expression of PDGFR α , p-Src^{Y418}, and p-Dock180^{Y1811} was found in normal brain and WHO grade I glioma specimens (Supplemental Figure 11 and data not shown). Spearman's rank correlation analysis of the expression of PDGFR α and p-Dock180^{Y1811} in clinical glioma specimens by IHC staining corroborated the correlation coefficient between border and border areas ($r^2 = 0.9000$; $P < 0.05$), center and center regions ($r^2 = 0.9000$; $P < 0.05$), and invasion and invasion areas ($r^2 = 0.8721$; $P < 0.05$) (Supplemental Tables 3 and 4).

To further validate these findings, we examined expression of PDGFR α , p-Src^{Y418}, and p-Dock180^{Y1811} by IB analyses in tumor lysates from a separate and independent cohort of 38 clinical GBM specimens. PDGFR α was overexpressed in 4 GBMs (tumors 4, 13, 14, and 30), and PDGFR α protein was detected at moderate to high levels in another 11 tumors (Figure 9G). Dock180 was detected at high levels in 25 of these 38 tumors, and p-Src^{Y418} and phosphorylated p21-activated kinase 1/2 (p-PAK1/2; activated by binding to p21-GTPases, including Rac1) were found in all tumors at different levels. Interestingly, p-Dock180^{Y1811} was coexpressed with p-Src^{Y418} and p-PAK1/2 in 8 PDGFR α -expressing GBMs (Figure 9G), which suggested activation of Src/Dock180/Rac1 signaling in these PDGFR α -expressing GBMs. Kaplan-Meier analysis showed that patients with high levels of both PDGFR α and p-Dock180^{Y1811} had significantly shorter overall survival compared with those with low PDGFR α and p-Dock180^{Y1811} (Figure 9, H and I). Finally, we searched for p-Dock180^{Y1811} in proteomic studies of p-Y proteins in human cancers and found that p-Dock180^{Y1811} has previously been detected in clinical specimens and cell lines of various types of human cancers, including gliomas, lung, breast, bladder, ovarian, oropharyngeal, and nonmelanoma skin cancers (<http://www.phosphosite.org> and ref. 25). Taken together, these data suggest that Dock180 is found

phosphorylated at its Y1811 site, and p-Dock180^{Y1811} is coexpressed with PDGFR α and p-Src^{Y418} in clinical human glioma specimens and could be a clinically useful marker in the diagnosis and assessment of outcome in GBMs with PDGFR α overexpression.

Discussion

Here we report a mechanism by which Rac1, a key modulator of cell motility and growth, is activated by its GEF, Dock180, in PDGFR α -promoted glioma tumorigenesis (Figure 10). We found that Dock180 was not only required for PDGFR α -promoted glioma cell growth, survival, and invasion *in vitro* and *in vivo*, but was also specifically p-Y at Y1811 in an Src-dependent manner. p-Dock180^{Y1811} mediated PDGFR α stimulation of glioma tumorigenesis through association of Dock180 with CrkII and p130^{Cas} and activation of Rac1. Additionally, Dock180 was associated with the PDGFR α receptor itself upon PDGF-A stimulation, and, without affecting induced p-Dock180^{Y1811}, disruption of Dock180 association with PDGFR α impeded PDGFR α -promoted glioma cell migration. Furthermore, p-Dock180^{Y1811} and p-Src^{Y418} were coexpressed with PDGFR α in clinical glioma specimens, and p-Dock180^{Y1811} was detected in several types of human cancers, including gliomas. Additionally, expression of p-Dock180^{Y1811} and PDGFR α correlated with a very poor clinical prognosis in patients with gliomas. Taken together, our results suggest a critical role of activation of p-Dock180^{Y1811}/Rac1 signaling in promoting cancer tumorigenesis.

Genetic studies have established a pathway of Dock180/CrkII/Rac1 in *C. elegans* (11) and placed Dock180/Rac1 downstream of PVR (the homolog of PDGFR/VEGFR) in *Drosophila* (14, 26). In the present study, we further established that PDGF-A stimulation of PDGFR α induced Src-dependent p-Y at Dock180^{Y1811}, leading to the formation of the Dock180/CrkII/p130^{Cas} complex and activation of Rac1 signaling and thereby promoting glioma cell growth, survival, and invasion (Figure 10). Since Rac1 is a direct downstream target of Dock180 (11) and mediates cancer cell growth, survival, and motility (10, 15, 27), inhibition of Dock180 by siRNA knockdown or reversion with Dock180^{Y1811F*} abrogated PDGFR α -stimulated Rac1 activity and tumorigenic behaviors of glioma cells *in vitro* and *in vivo*. Moreover, inhibition of Dock180 attenuated PDGFR α activation of p-Erk1/2, but with less reduction of p-Akt. We recently demonstrated that in glioblastomas deficient in *Ink4a/Arf*, overexpressed PDGFR α promotes tumorigenesis through the PI3K/Akt/mTOR-mediated pathway regulated by SHP-2 (8). Since both PDGFR α /PI3K signaling and PDGFR α /Src/Dock180/Rac1 signaling stimulate p-Akt, inhibition of Dock180 only partially reduced p-Akt, but attenuated PDGFR α -promoted survival and growth of glioma cells. On the other hand, inhibition of PI3K by LY294002 did not affect PDGF-A-induced p-Y of PDGFR α and p-Y of Dock180, but abrogated PDGF-A stimulation of Rac1 activities and cell migration, corroborating a recent study showing that PI3K is upstream of Rac1 in PDGFR α -induced cell migration (21). Additionally, the requirement for both PI3K/Akt/SHP-2/mTOR and Src/Dock180/Rac1 signaling in PDGFR α -promoted glioma tumorigenesis (ref. 8 and the present study) recapitulates the heterogeneity of glioblastomas that engenders their malignancy through multiple pathways. This hypothesis is further supported by our data showing that activated PAK1/2 (p-PAK1/2) and active Src (p-Src^{Y418}) were found in each of the 38 clinical GBM specimens analyzed, whereas active Dock180 (p-Dock180^{Y1811}) was only observed in 10 of them. PAK1/2 is activated by several p21-GTPases, including Rac1 and Cdc42 (9). We also made a similar observation in our IHC, studies in which p-Dock180^{Y1811} was absent in a number of glioma samples that



express PDGFR α and/or p-Src^{Y418}. Since PDGFR α and p-Src^{Y418} are activated in glioblastomas (2, 3, 28), GBMs that lack p-Dock180^{Y1811} might use alternative signaling pathways for their tumorigenesis. Taken together, our results not only corroborated with genetic studies showing that Dock180/Rac mediates cell migration induced by PDGFR (PVR in *Drosophila*) (14), but also integrate PDGFR α activation of Src into p-Dock180^{Y1811}/Rac1-promoted glioma cell growth, survival, and invasion (Figure 10).

Dock180 was identified as a binding protein for c-Crk through its C-terminal PxxP region (29). The PxxP domain-mediated formation of a Dock180/CrkII/p130^{Cas} complex is required for integrin stimulation of Rac1 and cell motility (11). Importantly, Y1811 is located within this PxxP domain (Figure 3B), which is highly conserved in Dock180 from opossum to humans, but not in other members of the Dock family (Figure 3F and Supplemental Figure 2). Since Dock180 activity is regulated by a conformational change upon ELMO1 association (11), it is possible that the PDGFR α -induced p-Dock180^{Y1811} at its C-terminal PxxP domain and formation of the Dock180/CrkII/p130^{Cas} complex caused a further conformation change, resulting in increased Dock180 binding to Rac1 and Rac1 activation. This provides a rationale for the observed biological and biochemical consequences of the specific p-Dock180^{Y1811} in PDGFR α -stimulated gliomagenesis. Furthermore, overexpression of PDGFR α in clinical GBMs (4), co-overexpression of p-Dock180^{Y1811} with PDGFR α and p-Src^{Y418} in clinical glioma specimens, and the occurrence of p-Dock180^{Y1811} in several types of human cancers (ref. 25 and <http://www.phosphosite.org>) suggest that p-Dock180^{Y1811} could be specifically responsible for the activation of Rac1 that promotes tumorigenesis of human cancers.

PDGF induces Src association with PDGFR α at a specific p-Y docking site in the receptor (30). Moreover, upon stimulation of RTKs, Src induces p-Y of several GEFs, such as Vav2 (10), which suggests that Src-dependent p-Y of GEFs could be a mechanism in RTK-promoted tumorigenesis. Our data support this hypothesis. In contrast to a previous report of p-Y of ELMO1 by Src family kinase Hck (31), we did not detect p-Y of ELMO1 upon PDGFR α activation. However, p-Dock180^{Y1811} was markedly induced in PDGF-A-stimulated glioma cells and primary GBM5 cells with PDGFR α overexpression, and inhibition of Src impaired PDGFR α -induced p-Dock180^{Y1811}. In silico analyses identified Y1811 and other potential p-Y sites in Dock180 as Src substrate sites. Our data validated Y1811 as a PDGFR α -induced Src p-Y site. Additionally, there was a minor p-Y site of Dock180 that was induced by PDGFR α activation (Figure 3E), but was not affected by Src inhibitors (Figure 6A), suggestive of a Src-independent p-Y of Dock180 not involved in PDGFR α -stimulated cell migration (Figure 6B). Based on these data, it would be predicted that Src family kinase inhibitors such as Dasatinib or AZD0530 (20) could be effective to inhibit PDGFR α -promoted glioma tumorigenesis in the brains of animals. However, caution is in order with this idea, since the Src family kinase inhibitors PP2 and SU6656 were previously shown to have minimal or moderate impact on PDGFR α -stimulated anchorage-independent growth of human glioma cells in vitro (8).

Multiple Rho GEFs interact with RTKs through various functional domains, affecting their GEF activities (12). We showed that PDGF-A induced an association of Dock180 with PDGFR α through its N-terminal domain (1–159 aa residues), which was critical for PDGFR α -stimulated Rac1 activity and glioma cell migration. The interaction region of Dock180 contains a SH3 domain that binds to ELMO1. Reexpression of shRNA-resistant Dock180^{A160*}, but not Dock180^{WT*},

in LN444/PDGF-A/shDock180 cells resulted in loss of interaction among Dock180^{A160*}, PDGFR α , and ELMO1, loss of Rac1 activity, and decreased glioma cell migration without affecting induction of p-Y of Dock180. Additionally, a double Dock180^{A160-Y1811F*} mutant showed an additive effect on the inhibition of PDGFR α stimulation. Our data could explain the previous observation that overexpression of Dock180 lacking the DHR-1 domain – which is a PtdIns(3,4,5)P3-binding domain – leads to activation of Rac1 (32) and does not require ELMO1 for this activity (11). We speculate that PDGF-A induces association of Dock180 with PDGFR α through the N-terminal region (1–159 aa residues) of Dock180, which probably is adjacent to or overlaps with the SH3 domain that binds to ELMO1, thereby opening the inhibitory folding configuration of Dock180 in unstimulated cells (11). Interaction of Dock180 with PDGFR α could additionally target Dock180 to the membrane in synergy with the DHR-1 domain, inducing formation of the Dock180/CrkII/p130^{Cas} complex and stimulating Rac1 activities and cell motility (11). These data also support the hypothesis that DHR-1 plays a role in dynamic membrane targeting of the Rho GEF activity of Dock180 in PDGFR α -activated cells (33). On the other hand, we found that association of Dock180 to PDGFR α was independent of Src-induced p-Dock180^{Y1811}, since the Dock180^{Y1811F} mutant was still able to bind to PDGFR α upon PDGF-A stimulation. Moreover, disruption of Dock180 binding to PDGFR α , a single Dock180^{Y1811F} mutant, or a double Dock180^{A160-Y1811F*} mutant abrogated PDGF-A activation of Rac1 and cell motility. The requirement of these 2 separate mechanisms fits well with a hypothetical 2-step model of bipartite GEF activation (11). Namely, in addition to association with ELMO1, PDGF-A-induced binding of Dock180 to PDGFR α targets Dock180 to the membrane, thereby facilitating interaction of ELMO1 and Dock180 with nucleotide-free Rac1. Since Y1811 is located farther away from the N-terminal SH3 domain of Dock180 and may not involve in the interaction of Dock180 with Rac1, it is possible that the PxxP domain with unphosphorylated Dock180^{Y1811} could hinder the loading of GTP into nucleotide-free Rac1. Src-induced p-Dock180^{Y1811} and formation of the Dock180/CrkII/p130^{Cas} complex caused a further conformation change of Dock180, thereby allowing GTP loading to nucleotide-free Rac1 and resulting in activation of Rac1 signaling and various cellular functions. However, this hypothesis warrants further investigation.

In summary, our data reveal a mechanism by which PDGFR α stimulates glioma tumorigenesis through PDGFR α -induced Src-dependent p-Y of Dock180^{Y1811} as well as Dock180 association with activated PDGFR α , thereby activating the Dock180/CrkII/p130^{Cas}/Rac1 pathway. This is underscored by coexpression of p-Dock180^{Y1811} and p-Src^{Y418} with overexpressed PDGFR α in clinical glioma specimens and a notable association with very poor patient survival. It also fits well with the occurrence of p-Dock180^{Y1811} that is detected by proteomic analysis in various types of human cancers, including gliomas. Since p-Y of Rho GEFs is a common mechanism affecting GEF activity, and Src is aberrantly activated in human cancers, including gliomas, our study suggests that the PDGFR α /Src/p-Dock180^{Y1811}/Rac1 signaling axis could represent a novel and attractive therapeutic target for glioblastomas and other types of human cancer that overexpress PDGFR α .

Methods

Cell lines and reagents. HEK293T, glioma U87, U251, U373, and T98G cells (all from ATCC); SNB19, LN2308, LN18, LN235, LN319, LN443, and LN444 cells; and unaltered primary human GBM cells were cultured as previously described (15, 24, 34). Rabbit polyclonal antibodies



to p-Dock180^{Y1811} were produced by immunizing animals with a synthetic phosphopeptide corresponding to residues surrounding human Dock180^{Y1811}. The antibodies were then affinity purified. YOP PTP was from Enzo Life Science; recombinant Src protein was from Active Motif; and lentivirus-encoded Src shRNAs were from The Broad Institute. Flag-Dock180 was provided by M. Matsuda (Kyoto University, Kyoto, Japan); PDGFR α ^{A8,9} by I. Clarke (University of Toronto, Toronto, Ontario, Canada); PDGF-A and PDGFR α by C.-H. Heldin (Uppsala University, Uppsala, Sweden); CrkII by R. Birge (UMDNJ–New Jersey Medical School, Newark, New Jersey, USA); Src-WT, -KD, and -CA by S. Courtneidge (Sanford-Burnham Medical Research Institute, La Jolla, California, USA); pMXI-*gfp* by R. Pieper (UCSF, San Francisco, California, USA); and SNB19 cells by Y. Zhou (UCI, Irwin, California, USA).

Purification of recombinant proteins. Protein purifications were performed at 4°C. (His)₆-tagged Dock180^{WT} and Dock180^{Y1811F} proteins were purified from serum-starved HEK293T cells transiently transfected with pcDNA3-(His)₆-Dock180^{WT} and -Dock180^{Y1811F}, respectively. Cells were lysed and sonicated. The lysates were centrifuged, and the supernatants were loaded onto a Ni²⁺-NTA column in 10 mM imidazole buffer. After washing 2 \times , Ni²⁺-bound proteins were eluted with a 500 mM imidazole buffer followed by dialysis against PBS. The purified recombinant Dock180 proteins were examined by Coomassie blue staining and IB analyses. The aliquots were stored at 80°C until use.

In vitro Src p-Y and Rac1/Dock180 binding assays. 500 ng purified recombinant Dock180^{WT} or Dock180^{Y1811F} proteins were incubated with 200 μ M cold ATP in the presence or absence of 100 ng recombinant active Src kinase (Active Motif) in 30 μ l reaction buffer (60 mM Hepes-NaOH, pH 7.5, 3 mM MnCl₂, 3 mM MgCl₂, 3 mM Na₂VO₄, 1.2 mM DTT, 1.5 μ g PEG 20,000) at 30°C for 30 minutes and then chilled on ice. The reaction products were mixed with an equal volume of 2 \times SDS sample buffer and p-Dock180^{Y1811}, then examined by IB analyses using the specific anti-p-Dock180^{Y1811} antibody.

The effect of Src-induced p-Dock180^{Y1811} on Rac1 binding was determined as previously described (22, 23). Briefly, Flag-tagged Dock180^{WT} or Dock180^{Y1811F} cDNAs were transfected into HEK293T cells for 48 hours. Cells were then lysed, and Flag-tagged Dock180^{WT} or Dock180^{Y1811F} proteins were subjected to IP using an anti-Flag antibody. The precipitates were then treated with 15 μ M of a recombinant YOP PTP at 30°C for 1 hour in 1 \times YOP reaction buffer (50 mM citrate, pH 6.0, 100 mM NaCl, 1 mM EDTA, and 1 mM DTT) containing 1 mg/ml BSA, washed 3 \times with PBS, and incubated with or without a recombinant Src kinase at 30°C for 30 minutes. The treated mixtures were washed again and incubated with total lysates prepared from HEK293T cells that were transfected with a EGFP-Rac1 cDNA with 10 mM EDTA at 4°C for 90 minutes. The reaction products were mixed with an equal volume of IP buffer or 2 \times SDS sample buffer and examined by IP and IB analyses.

IHC and IB analyses of human and mouse glioma specimens. In total, 134 primary human glioma specimens were collected from 2001 to 2008 at Saitama Medical University and Kyorin University. Specimens were examined and diagnosed by a neuropathologist, then analyzed by IHC using an anti-PDGFR α antibody (diluted 1:20) as we previously described (15). IHC analyses were further performed on 33 specimens that were positive for PDGFR α expression and 9 samples that were negative for PDGFR α expression using specific anti-p-Dock180^{Y1811} and anti-p-Src^{Y418} antibodies (diluted 1:20 and 1:10, respectively). Mouse brain sections with various tumors were analyzed by IHC using an anti-Ki-67 antibody (diluted 1:200) or a TUNEL staining kit. Images were captured using a microscope equipped with a digital camera. 5 random images per section of mouse brains were obtained, and percentage of Ki-67- or TUNEL-positive cells was quantified and statistically analyzed as previously described (8).

A separate and independent cohort of a total of 38 snap-frozen WHO grade IV GBM tissue samples were obtained from the University of Pittsburgh Medical Center Tissue Bank. These tissue samples were neurological specimens discarded as excess tissues and collected and banked by the Tissue Bank at the University of Pittsburgh; tumors were examined and diagnosed by a neuropathologist. The small frozen fragments of tumor tissues (0.07–0.9 g) were processed and analyzed by IB assays (8).

Gene knockdown by siRNA or shRNA; mutagenesis, IP, IB, cell proliferation, and viability assays; in vitro cell migration and Rac1 activation assays; mouse glioma xenografts; gene expression analysis; and quantitative PCR analysis for gene copy numbers. See Supplemental Methods.

Statistics. GraphPad Prism version 4.00 for Windows was used to perform 1-way ANOVA with Newman-Keuls post-test or paired 2-tailed Student's *t* test and χ^2 test as previously described (15). A *P* value of 0.05 or less was considered statistically significant.

Study approval. Studies using human tissues were reviewed and approved by the Institutional Review Board involving Human Subjects of the University of Pittsburgh. The specimens were deidentified human tissues; thus, no informed consent was required.

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