PHLDA1 promotes glioblastoma cell growth via sustaining the activation state of Ras

Jiutao Wang1,2 · Ning Yao1 · Yamei Hu1 · Mingjuan Lei1,2 · Meixian Wang2 · Lu Yang1 · Satyananda Patel2 · Xiang Li1,2 · Kangdong Liu1,2 · Zigang Dong1,2

Received: 20 June 2022 / Revised: 9 August 2022 / Accepted: 29 August 2022 / Published online: 15 September 2022
© The Author(s), under exclusive licence to Springer Nature Switzerland AG 2022

Abstract
Activation of the Ras signaling pathway promotes the growth of malignant human glioblastoma multiforme (GBM). Mutations in Ras are rare in GBM, elevated levels of activated Ras are prevalently observed in GBM. However, the potential mechanism of how Ras is activated in GBM remains unclear. In this study, we screened a new interacted protein of Ras, PHLDA1. Our findings confirmed that PHLDA1 acted as an oncogene and promoted glioma progression and recurrence. We demonstrated that PHLDA1 was upregulated in GBM tissues and cells. PHLDA1 overexpression promoted cell proliferation and tumor growth. In terms of mechanism, PHLDA1 promoted cell proliferation by regulating Ras/Raf/Mek/Erk signaling pathway. Moreover, Src promotes GTPase activity of Ras via tyrosine 32 phosphorylation. PHLDA1 and Src competed for binding with Ras, inhibiting Ras phosphorylation by Src and rescuing Ras activity. This study may provide a new idea of the molecular mechanism underlying glioma progression and a novel potential therapeutic target for comprehensive glioblastoma treatment.

Keywords Ras · PHLDA1 · Src · Glioblastoma multiforme · Ras phosphorylation

Introduction
GBM is the most common and highly aggressive malignant brain tumor in humans. GBMs are classified as either primary or secondary GBM [1]. Primary GBM, which comprises around 90% of all GBMs, develops rapidly de novo, whereas secondary GBM progresses from a low-grade astrocytoma to a high-grade glioma through the acquisition of additional genetic alterations [2]. In both cases, hyperactive receptor tyrosine kinases, epidermal growth factor receptor (EGFR) and platelet-derived growth factor receptor (PDGFR) potentiate common downstream Ras and phosphatidylinositol 3-kinase/Akt pathways to drive tumor growth and survival [3, 4]. Activation of Ras signals is a critical event in many cancers. Mutations in codon 12 or 61 are the most frequent way for activating Ras. However, in certain cancers Ras was highly activated without Ras mutation, like GBM [5, 6]. To understand how Ras was activated in these cancers is not only an interesting scientific question but also an extreme need for the therapy of these cancers.

Here, a new interacted protein of Ras was screened, Pleckstrin homology-like domain family A member 1 (PHLDA1), which plays an oncogenic role in many cancers. PHLDA1 encodes a 401-amino acid protein that comprises a central pleckstrin homology domain like domain family A member 1 (PHLDA1), which plays an oncogenic role in many cancers. PHLDA1 encodes a 401-amino acid protein that comprises a central pleckstrin homology domain common to proteins involved in intracellular signaling [7–9], a central polyglutamine tract, and 2C-terminal regions rich in proline glutamine and proline-histidine repeats. The gene is expressed in a wide range of normal and cancer tissues [10, 11], suggesting that it may have important cellular functions [12, 13]. PHLDA1 expression is induced by external stresses such as heat shock [14, 15], and may be modulated by insulin-like growth factor I (IGF-I) [13] and extracellular-regulated kinase (ERK) pathways [15]. In addition, PHLDA1 subcellular localization varies. PHLDA1 is found predominantly in the cytoplasm [10, 11, 16, 17]. However, perinuclear, nucleolar and nuclear localization have also been reported [13, 16–18]. PHLDA1...
is associated with different biological processes, such as cell apoptosis [19], cell proliferation [20] and differentiation [21].

Highly activated Ras plays a critical role in GBM without Ras mutation. Here, we reported a novel mechanism of Ras activation in GBM. PHLDA1 promotes Ras activity via inhibiting Src tyrosine kinase phosphorylates Ras, and then activate downstream proliferative Raf/Mek/Erk signaling. These results identify PHLDA1 as an activator of Ras and a potential therapeutic target for cancers driven by a previously ‘undruggable’ oncogenic or hyperactive Ras.

Results

PHLDA1 accumulates in glioma tissues

Using PHLDA1-specific antibody, we performed IHC analysis on a large cohort of glioma patients, the representative images showed both low expression of PHLDA1 in normal brain and high expression of PHLDA1 in two glioma patient brains (Fig. 1A). We quantified the expression of PHLDA1 in the normal human brain (n = 3), low grade (n = 97) and high grade (n = 79) glioma brains, the results indicated that expression level of PHLDA1 was higher in different grade glioma tissue than that of normal tissue samples (Fig. 1B). The expression level of PHLDA1 was higher in female compared with male patients (Fig. 1C). Quantification of PHLDA1 histological staining enabled us to identify two subgroups of patients characterized either by low or high PHLDA1 protein levels, using the median PHLDA1 histological score (Hscore) as a cut-off. Interestingly, PHLDA1 protein levels exhibited a prognostic value, as overall survival was markedly shortened in patients whose tumors exhibited high PHLDA1 protein levels (Fig. 1D). Moreover, the disease-free survival time was dramatically decreased in high PHLDA1 patients than low PHLDA1 patients (Fig. 1E). Patients with high PHLDA1 levels showed a higher tendency of recurrence than a low expression of PHLDA1 patients (Fig. 1F). In addition, we analyzed the correlation between PHLDA1 and other markers of glioblastoma. In accordance with the Kaplan–Meier survival results, Pearson Correlation

![Fig. 1 PHLDA1 accumulates in glioma tissues. A Representative images of H&E and IHC staining showed low expression of PHLDA1 in normal brain and high expression of PHLDA1 in two glioma patient brains. Scale bars, 100 μm. B Quantification of PHLDA1 expression in 3 non-tumorous and 176 glioma samples was assessed by immunohistochemistry. The integrated optical density (IOD) was evaluated using the Image-Pro premier software offline (v9.0) program. C Quantification of PHLDA1 expression in male and female patients. D–F Kaplan–Meier plot of Overall survival (D), Disease-free survival (E) and recurrence rate (F) of glioma patients stratified by PHLDA1 protein levels. *P < 0.05 and **P < 0.01](image-url)
is significantly related between PHLDA1 and overall survival, as the other clinical marker PCNA and ki67 (Table 1). Thus, our results indicate that PHLDA1 protein is a new prognostic marker that might be an interesting therapeutic target in glioma.

**PHLDA1 promotes glioblastoma cell proliferation in vitro**

According to the UCSC genome database, *PHLDA1* gene encodes long and short isoforms of PHLDA1 protein. The molecular weight of PHLDA1-long and short are 45 and 30 kDa, but the bands appear at 53 and 38 kDa respectively [22]. The PH domain of PHLDA1 is a so-called split PH domain in which the PH domain is divided into N-terminal (β sheets 1–3) and C-terminal (β sheets 4–7 and an α helix) portions (Fig. 2A). In consistent with the previous study, we demonstrated in this study that short form of PHLDA1 protein was predominantly expressed in human cells, the endogenous PHLDA1 protein was much smaller, with a size of approximately 38 kDa. The western blot results showed that PHLDA1 was relatively low expressed in HEB and LN229 cells, while highly expressed in U87 and U251 cells (Fig. 2B). In the following work, to check how PHLDA1 regulated cell phenotypes, we knocked down PHLDA1 in U87 and U251 cells and overexpressed in HEB and LN229 cells. To examine whether PHLDA1 can affect glioblastoma cell proliferation, two different shPHLDA1 sequences were designed to establish PHLDA1 knockdown U87 and U251 cells, and the protein abundance of PHLDA1 was greatly decreased in shPHLDA1 cells compared with control cells (Fig. 2C). Next, we used MTT and soft agar colony formation assays to assess whether PHLDA1 had effects on cell proliferation and colony formation in glioblastoma. Our results indicated that knockdown of PHLDA1 slowed cell growth and lead to the formation of fewer colonies in both knockout mouse brain cells (Fig. 3A) and PHLDA1 knockdown cells (Fig. 3B and C). To further confirm that PHLDA1 enhances cell proliferation through the Ras signaling pathway, HEB and LN229 s transiently transfected with the HA-PHLDA1 and treated with or without HRas inhibitor (kobe0065). Western blotting revealed that phosphorylation of c-raf was up-regulated with PHLDA1 overexpression in both cell lines, and that the up-regulations could be inhibited by kobe0065 treatment in a dose-dependent manner (Fig. 3D). Consistent with the results of kobe0065 treatment, phosphorylation of Erk1/2 and p90RSK were up-regulated with PHLDA1 overexpression in both cell lines, and that the up-regulations could be inhibited by combinetinib treatment (Fig. 3E). In addition, immunofluorescence images also confirmed that the nuclear translocation of phosphorylated CREB in accompany with PHLDA1 upregulation (Fig. 3G). These data suggest that PHLDA1 enhances cell proliferation through the Ras/Raf/Mek/Erk pathway.

**PHLDA1 promotes Ras activity via inhibiting Ras phosphorylation by Src tyrosine kinase**

The above results prompted us to examine the effect of PHLDA1 on Ras activity. We thus measured the activity of Ras in the cell lysates with either overexpression or knockdown of PHLDA1 using an active Ras pull-down and detection kit. GST-fusion protein of the Ras-binding domain (RBD) of Raf1 along with glutathione agarose resin were used to specifically pull-down active Ras and an anti-Ras antibody for western blot detection. Western blot results showed that Ras activity was significantly increased upon overexpression of PHLDA1 in HEK293T cells (Fig. 4A), while slightly increased in U87 cells which is predominantly in the active GTP-bound form, whereas silencing of PHLDA1 decreased Ras activity (Fig. 4B and C). To explore the potential molecular mechanism between PHLDA1 and Ras, immunoprecipitation assays were performed to check whether PHLDA1 can interact with Ras. The results indicated that endogenous PHLDA1 can precipitate endogenous Ras in U87 cells and vice versa (Fig. 4D). To further determine which domains of PHLDA1 differentially contributed to the interaction with Ras, we constructed a series of vectors with different PHLDA1 domain deletions. The immunoprecipitation results showed that Ras precipitated full length and PQ domain of PHLDA1 (Fig. 4E). These data suggest that the PQ domain rather than the PH domain of PHLDA1 was essential for the association with Ras. Previous studies demonstrated that Src tyrosine kinase directly phosphorylates Ras on tyrosine 32 within the switch I region [24].

Ras and Raf are key mediators in one of the major signal-transduction pathways that regulate cell proliferation, the Ras/Raf/Mek/Erk pathway [23]. To examine the effect of PHLDA1 on Ras signaling, we analyzed the phosphorylation status of Erk1/2, p90RSK and transcriptional factor CREB in PHLDA1 knockout mouse and U87 and U251 cells with knockdown of PHLDA1. Immunoblotting results showed that Erk1/2, p90RSK and CREB phosphorylation were downregulated in both knockout mouse brain cells (Fig. 3A) and PHLDA1 knockdown cells (Fig. 3B and C). To further confirm that PHLDA1 enhances cell proliferation through the Ras signaling pathway, HEB and LN229 s transiently transfected with the HA-PHLDA1 and treated with or without HRas inhibitor (kobe0065). Western blotting revealed that phosphorylation of c-raf was up-regulated with PHLDA1 overexpression in both cell lines, and that the up-regulations could be inhibited by kobe0065 treatment in a dose-dependent manner (Fig. 3D). Consistent with the results of kobe0065 treatment, phosphorylation of Erk1/2 and p90RSK were up-regulated with PHLDA1 overexpression in both cell lines, and that the up-regulations could be inhibited by combinetinib treatment (Fig. 3E). In addition, immunofluorescence images also confirmed that the nuclear translocation of phosphorylated CREB in accompany with PHLDA1 upregulation (Fig. 3G). These data suggest that PHLDA1 enhances cell proliferation through the Ras/Raf/Mek/Erk pathway.

**PHLDA1 activates Ras/Raf/Mek/Erk signaling pathway**

Ras and Raf are key mediators in one of the major signal-transduction pathways that regulate cell proliferation, the Ras/Raf/Mek/Erk pathway [23]. To examine the effect of PHLDA1 on Ras signaling, we analyzed the phosphorylation status of Erk1/2, p90RSK and transcriptional factor CREB in PHLDA1 knockout mouse and U87 and U251 cells with knockdown of PHLDA1. Immunoblotting results showed that Erk1/2, p90RSK and CREB phosphorylation were downregulated in both knockout mouse brain cells (Fig. 3A) and PHLDA1 knockdown cells (Fig. 3B and C). To further confirm that PHLDA1 enhances cell proliferation through the Ras signaling pathway, HEB and LN229 s transiently transfected with the HA-PHLDA1 and treated with or without HRas inhibitor (kobe0065). Western blotting revealed that phosphorylation of c-raf was up-regulated with PHLDA1 overexpression in both cell lines, and that the up-regulations could be inhibited by kobe0065 treatment in a dose-dependent manner (Fig. 3D). Consistent with the results of kobe0065 treatment, phosphorylation of Erk1/2 and p90RSK were up-regulated with PHLDA1 overexpression in both cell lines, and that the up-regulations could be inhibited by combinetinib treatment (Fig. 3E). In addition, immunofluorescence images also confirmed that the nuclear translocation of phosphorylated CREB in accompany with PHLDA1 upregulation (Fig. 3G). These data suggest that PHLDA1 enhances cell proliferation through the Ras/Raf/Mek/Erk pathway.
| Table 1  | Correlation each clinicopathological characteristic of GBM |
|----------|------------------------------------------------------------|
|          | Overall survival time | Recurrence time | PHLDA1 | ki67 | GFAP | S100 | PCNA | Vimentin | Gender | Age | Grade |
| Pearson Correlation | 1 | 0.796** | -0.153* | -0.465** | 0.022 | 0.150 | -0.214* | 0.107 | 0.097 | -0.139 | -0.671** |
| Sig. (2-tailed) | 0.000 | 0.043 | 0.000 | 0.786 | 0.065 | 0.041 | 0.457 | 0.199 | 0.068 | 0.000 |
| N | 175 | 175 | 72 | 159 | 151 | 92 | 51 | 175 | 174 | 175 |
| Recurrence time | 0.796** | 1 | -0.254** | -0.473** | -0.076 | 0.150 | -0.305** | 0.150 | 0.116 | -0.197** | -0.661** |
| Pearson Correlation | 0.000 | 0.001 | 0.000 | 0.342 | 0.066 | 0.003 | 0.295 | 0.125 | 0.009 | 0.000 |
| Sig. (2-tailed) | 175 | 175 | 72 | 159 | 151 | 92 | 51 | 175 | 174 | 175 |
| N | -0.153* | -0.254** | 1 | 0.064 | 0.115 | -0.021 | 0.175 | 0.224 | 0.153* | 0.247* | 0.174* |
| PHLDA1 | 0.043 | 0.000 | 0.590 | 0.638 | 0.900 | 0.054 | 0.468 | 0.713 | 0.211 | 0.000 |
| N | 72 | 72 | 72 | 71 | 65 | 14 | 39 | 72 | 72 | 72 |
| ki67 | -0.465** | -0.473** | 0.064 | 1 | 0.057 | 0.016 | 0.526 | -0.120 | -0.044 | 0.149 | 0.482** |
| Pearson Correlation | 0.000 | 0.000 | 0.590 | 0.638 | 0.900 | 0.054 | 0.468 | 0.713 | 0.211 | 0.000 |
| Sig. (2-tailed) | 0.022 | 0.006 | 0.342 | 0.150 | 0.305** | 0.235 | 0.251 | 0.003 | 0.914 | 0.363 | 0.904 |
| N | 72 | 72 | 72 | 71 | 65 | 14 | 39 | 72 | 72 | 72 |
| GFAP | 0.022 | -0.076 | 0.115 | 0.057 | 1 | 0.098 | 0.121 | 0.408** | 0.009 | 0.073 | 0.010 |
| Pearson Correlation | 0.786 | 0.342 | 0.150 | 0.638 | 0.305** | 0.235 | 0.251 | 0.003 | 0.914 | 0.363 | 0.904 |
| Sig. (2-tailed) | 0.159 | 0.159 | 0.71 | 159 | 149 | 92 | 50 | 159 | 158 | 159 |
| N | 72 | 72 | 72 | 71 | 65 | 14 | 39 | 72 | 72 | 72 |
| S100 | 0.150 | 0.150 | -0.021 | 0.016 | 0.098 | 1 | 0.148 | 0.123 | -0.039 | 0.039 | -0.094 |
| Pearson Correlation | 0.065 | 0.066 | 0.796 | 0.900 | 0.235 | 0.169 | 0.409 | 0.632 | 0.634 | 0.250 |
| Sig. (2-tailed) | 151 | 151 | 151 | 65 | 149 | 88 | 47 | 151 | 150 | 151 |
| N | 151 | 151 | 151 | 65 | 149 | 88 | 47 | 151 | 150 | 151 |
| PCNA | 0.214* | -0.305** | 0.175 | 0.526 | 0.121 | 0.148 | 1 | -0.254 | -0.062 | 0.099 | 0.297** |
| Pearson Correlation | 0.041 | 0.003 | 0.096 | 0.054 | 0.251 | 0.169 | 0.402 | 0.559 | 0.351 | 0.004 |
| Sig. (2-tailed) | 92 | 92 | 92 | 14 | 92 | 88 | 92 | 13 | 92 | 91 |
| N | 92 | 92 | 92 | 14 | 92 | 88 | 92 | 13 | 92 | 91 |
| Vimentin | 0.107 | 0.150 | 0.224 | -0.120 | 0.408** | 0.123 | -0.254 | 1 | 0.039 | 0.186 | -0.189 |
| Pearson Correlation | 0.457 | 0.295 | 0.114 | 0.468 | 0.003 | 0.409 | 0.402 | 0.788 | 0.190 | 0.183 |
| Sig. (2-tailed) | 51 | 51 | 51 | 39 | 50 | 47 | 13 | 51 | 51 | 51 |
| N | 51 | 51 | 51 | 39 | 50 | 47 | 13 | 51 | 51 | 51 |
| Gender | 0.097 | 0.116 | 0.153* | -0.044 | 0.009 | -0.039 | -0.062 | 0.039 | 1 | 0.001 | -0.162* |
| Pearson Correlation | 0.199 | 0.125 | 0.043 | 0.713 | 0.914 | 0.632 | 0.559 | 0.788 | 0.986 | 0.032 |
| Sig. (2-tailed) | 175 | 175 | 175 | 72 | 159 | 151 | 92 | 51 | 176 | 175 | 176 |
tyrosine 32 phosphorylation has two important effects on Ras signaling: phosphorylation inhibits the binding of the effector protein Raf while increasing the binding of the GAP protein leading to enhanced GTP hydrolysis [25]. To further examine the detailed mechanism of how PHLDA1 enhances Ras activity. An immunoprecipitation assay with ectopically expressed PHLDA1, Ras and Src showed that Flag-tagged Ras precipitated HA-tagged PHLDA1 and Src in transfected HEK293T cells (Fig. 4F). As Src is a tyrosine kinase, we also demonstrate in this study that Src could phosphorylate HRas, but most intriguingly, PHLDA1 could inhibit Ras phosphorylated by Src tyrosine kinase. In addition, in vitro kinase assay also confirmed the result (Fig. 4G). These observations suggest that PHLDA1 promotes Ras activity via inhibiting its phosphorylation by Src.

PHLDA1 and Src compete binding with Ras

We hypothesized that PHLDA1 and Src competed binding with the same domain of Ras. As we know, there were two areas represented by the red box (switch I and switch II) undergo conformational changes depending on Ras binding to GDP or GTP (Fig. 5A). SwitchI interacted with GAPs and effectors, switchII interacted with GEFs. To verify this hypothesis, different Ras domain mutants were constructed. Immunoprecipitation assays were performed to check whether PHLDA1 and Src can interact with different Ras deletion mutants (Ras△SWI, Ras△SWII and Ras△SWI/II). The results showed that HA-tagged PHLDA1 can interact with Ras△SWI and Ras△SWII separately, but not Ras△SWI/II (Fig. 5B). Interestingly, HA-tagged Src can interact with Ras△SWI and Ras△SWII separately as well, but not Ras△SWI/II (Fig. 5B). These results indicated that PHLDA1 and Src both can bind with the same domain of Ras, that is why PHLDA1 inhibits Ras phosphorylated by Src.

PHLDA1 promotes cell proliferation via Ras signaling

To confirm PHLDA1 promotes cell proliferation via Ras signaling. We used MTT and plate colony formation assays to assess whether Ras can rescue the inhibition of cell proliferation and colony formation caused by silencing of PHLDA1. MTT results showed that knockdown of PHLDA1 slowed cell growth and lead to the formation of fewer colonies in U87 and U251 cells compared to control cells (Fig. 6A and B). As expected, overexpression of active Ras(G12V) significantly promoted cell growth and colony formation and rescued the reduction of cell viability caused by silencing of PHLDA1. To further confirm this result, PHLDA1 overexpressed treated with or without Ras inhibitor kobe0065 in HEB and LN229 cells. MTT results showed
that upregulation of PHLDA1 promoted cell growth and lead to the formation of more colonies in HEB and LN229 cells, while a significant reduction of cell viability and colony number caused by kobe0065 treatment, upregulation PHLDA1 did not alleviate the inhibition of cell proliferation caused by kobe0065. These data suggest that PHLDA1 promotes cell proliferation via Ras signaling.

**Knockdown of PHLDA1 reduced tumor formation in vivo**

To evaluate whether PHLDA1 knockdown could inhibit the proliferation of U87 and U251 cells. U87-bearing xenograft mice model were established. U87 were a stable expression of green fluorescent protein (GFP) using a vector encoding enhanced GFP (pEGFP-C1). U87-GFP expressing cloned cells were transiently transfected with HA empty vector and HA-PHLDA1. The transfection efficiency of PHLDA1 was detected by Western blotting. Cell proliferation was measured upon PHLDA1 knockdown by MTT (D) and soft agar colony formation (E) assays. HEB and LN229 cells were transiently transfected with HA empty vector and HA-PHLDA1. The overexpression efficiency of PHLDA1 was detected by Western blotting. Cell proliferation was measured upon PHLDA1 overexpression by MTT (G) and soft agar colony formation (H) assays. *P < 0.05, **P < 0.01, ***P < 0.001. Error bars represent the mean ± SD from three independent experiments.
**PHLDA1 promotes glioblastoma cell growth via sustaining the activation state of Ras**

Fig. 3 PHLDA1 activates Ras/Raf/Mek/Erk signaling pathway. A Equal amounts of lysates were immunoblotted with the indicated antibodies from wild type and PHLDA1 knockout mouse brain. B and C U87 and U251 were stably infected with lentiviral scramble, shPHLDA1#1 or #2, equal amounts of lysates were immunoblotted with the indicated antibodies. D HEB and LN229 transiently transfected with the HA-PHLDA1 and treated with or without HRas inhibitor (kobe0065), followed by immunoblotting to detect indicated protein expression. E HEB and LN229 transiently transfected with the HA-PHLDA1 and treated with or without MEK inhibitor (cobimetitib), followed by immunoblotting to detect indicated protein expression. F U87 transiently transfected with the GFP-PHLDA1, the localizations of phosphorylated CREB were detected by confocal laser scanning microscopy as indicated. Arrows indicated PHLDA1-GFP positive cells, arrowheads indicated PHLDA1-GFP negative cells. Scale bar = 10 μm.
size remarkably decreased in mouse brain injected with U87 PHLDA1 depletion cells compared with that in the control group (Fig. 7C and D). The reduced proliferative nature of the tumor is consistent with the observed trend of increased survival at 60 days in PHLDA1 knockdown groups in comparison to the control group (Fig. 7E). To further determine the effect of PHLDA1 on glioma progression in vivo, a cell-derived xenograft (CDX) model was used, and lentivirus-mediated shPHLDA1 treatment was performed. The results showed that PHLDA1 knockdown in U87 markedly reduced CDX tumor volume and average tumor weight compared with those of the control group (Fig. 7F–H). Next, to further check whether the antitumor effect was associated with its inhibition of Ras signaling, tumor slices from each group were prepared and analyzed for the expression levels of PHLDA1, p-raf1 and Ki67. Immunohistochemistry analysis results showed that the PHLDA1 expression was reduced by lentiviral infection and that the expression of the cell proliferation marker Ki67 expression decreased. Furthermore, Ras downstream signals p-raf1 was reduced in the PHLDA1 inhibition group compared with that in the control group (Fig. 7I and J). These data provide strong evidence that inhibition of PHLDA1 suppresses tumor growth in vivo.

Discussion

Activation of Ras may be relevant to the proliferation of GBM cells and to tumor angiogenesis, both of which appear critical for the growth of human malignant GBM,
PHLDA1 promotes glioblastoma cell growth via sustaining the activation state of Ras

which are one of the most vascularized of human neoplasms [26, 27]. Although oncogenic mutations affecting Ras are not prevalent in human malignant GBM, but elevated levels of activated Ras activity are in human malignant GBM specimens. Previous study reported that elevated Ras-GTP levels in human malignant GBM cells result from proliferative signals emanating from RTKs such as the PDGFRs and EGFR, which are highly expressed in these tumor cells [28, 29]. However, the potential mechanism of how Ras is activated in GBM remains unclear. In the current study, we found a new interacted protein of Ras, PHLDA1. How PHLDA1 function as an oncogene in GBM remain unclear as well. In light of this, we proposed that PHLDA1 promoted glioma progression by regulating Ras activity and its downstream signaling pathway.

Previous study findings suggest that PHLDA1 has tissue-specific functions and may have an oncogenic role in certain cancer types [10, 18, 30–34]. Liu et al. confirmed that PHLDA1 promoted glioma progression [35]. In this study, we demonstrated that the expression of PHLDA1 was significantly increased in glioblastoma tissues and cell lines. PHLDA1 upregulation is correlated with the grade of the GBM and the gender of the patients. Positive expression of PHLDA1 protein was a strong predictor of poor prognosis for GBM patients, indicating an oncogenic role in GBM. To further determine the impact of PHLDA1 downregulation on cancer cell growth, we used lentivirus-based shRNA delivery to knock down PHLDA1 in GBM cells. Results show that knockdown of PHLDA1 significantly decreases proliferation of GBM cells. These data indicate that downregulation of PHLDA1 inhibits glioma progression and might act as a prognostic biomarker for GBM.

To explore the potential molecular mechanism between PHLDA1 and Ras, immunoprecipitation assays were performed to check whether PHLDA1 can interact with Ras. The results indicated that endogenous PHLDA1 could precipitate endogenous PHLDA1 in U87 cells and vice versa. To further determine which domains of PHLDA1 differentially contributed to the interaction with Ras, we constructed a series of vectors with different PHLDA1 domain deletions. The immunoprecipitation results showed that Ras precipitated full length and PQ domain of PHLDA1. These data suggest that the PQ domain rather than the PH domain of PHLDA1 was essential for the association with Ras. To reveal how PHLDA1 activated Raf/MAPK signaling pathway, we thus measured the activity of Ras that was pulled down from cell lysates with either overexpression or knockdown of PHLDA1. Western blot results showed that Ras activity was significantly increased upon overexpression of PHLDA1 in HEK293T cells, while slightly increased in U87 cells which is predominantly in the active GTP-bound form, whereas silencing of PHLDA1 decreased Ras activity. These findings suggested that PHLDA1 promoted cell proliferation by interacting with Ras and regulated its activity.

In an attempt to confirm that PHLDA1 promoted cell proliferation by regulating Ras activity, we knocked down and overexpressed PHLDA1 to detect if Ras downstream signaling pathway was affected or not. Immunoblotting results showed that Erk1/2, p90RSK and CREB phosphorylation were downregulated in both knockout mouse brain cells and PHLDA1 knockdown cells. To further confirm that PHLDA1 enhances cell proliferation through the Ras/Raf/Mek/Erk pathway. HEB and LN229 were transiently transfected with the HA-PHLDA1 and treated with or without HRas inhibitor (kobe0065). Western blotting revealed that

Fig. 5 PHLDA1 and Src compete binding with Ras. A Schematic representation of Ras domain structure showing switch I (SWI) domain and switch II (SWII) domain together with the amino acid number (a.a. #) range for each domain. RasΔSWI, Ras SWI domain deleted mutant; RasΔSWII, Ras SWII domain deleted mutant; RasΔSWI/II, Ras SWI and SWII domain deleted mutant. B HA immunoprecipitation (IP) from HEK293T cells expressing HA-tagged PHLDA1 together with the different Flag-tagged Ras domain mutants. C HA immunoprecipitation (IP) from HEK293T cells expressing HA-tagged Src together with the different Flag-tagged Ras domain mutants. Co-precipitated exogenous Ras mutants were detected by western blot analysis. GAPDH was used as a loading control. Representative western blot from three independent experiments.
phosphorylation of c-raf was up-regulated with PHLDA1 overexpression in both cell lines, and that the up-regulations could be inhibited by kobe0065 treatment in a dose-dependent manner. Consistent with the results of kobe0065 treatment, phosphorylation of Erk1/2 and p90RSK were up-regulated with PHLDA1 overexpression in both cell lines, and that the up-regulations could be inhibited by Mek inhibitor (combimetinib) treatment. In addition, immunofluorescence images also confirmed that the nuclear translocation of phosphorylated p90RSK in accompany with PHLDA1 upregulation. These data suggest that PHLDA1 enhances cell proliferation through the Ras/Raf/MEK/ERK pathway.

Previous studies demonstrated that Src tyrosine kinase directly phosphorylates Ras on tyrosine 32 and 64 within the switch I and II region [24]. This activity is particularly interesting since Tyr32 is located in the switch I region (aa 30–38) while Tyr64 is positioned in the switch II sequence of K-Ras (aa 59–76). These regions of Ras undergo conformational changes when GDP is exchanged for GTP. Ras tyrosine 32 phosphorylation inhibits the binding of the effector protein

Fig. 6 PHLDA1 promotes cell proliferation via Ras signaling. A Cell proliferation was measured using MTT. PHLDA1 knockdown combined overexpression with or without active Ras mutant (RasG12V) in U87 and U251, while PHLDA1 overexpression treated with or without Ras inhibitor kobe0065 in HEB and LN229 cells. B Plate colony formation assays were used to check cell proliferation. PHLDA1 knockdown combined overexpression with or without active Ras mutant (RasG12V) in U87 and U251, while PHLDA1 overexpression treated with or without Ras inhibitor kobe0065 HEB and LN229 cells. *P < 0.05, **P < 0.01. ***P < 0.001. Error bars represent the mean ± SD from three independent experiments.
Raf, while increases the binding of the GAP protein leading to enhanced GTP hydrolysis [36, 37]. We hypothesized that PHLDA1 and Src competed binding with the same domain of Ras. To verify this hypothesis, different Ras domain mutants were constructed. Immunoprecipitation assays were performed to check whether PHLDA1 and Src can interact with different Ras deletion mutants (RasΔSWI, RasΔSWII and RasΔSWI/II). The results showed that PHLDA1 and Src competed binding with the switch I and II domain, that is why PHLDA1 inhibits Ras phosphorylated by Src (Fig. 8). To further examine the detailed mechanism of how PHLDA1 enhances Ras activity. An immunoprecipitation assay with ectopically expressed PHLDA1, Ras and Src showed that Flag-tagged Ras precipitated HA-tagged PHLDA1 and Src in transfected HEK293T cells. As Src is a tyrosine kinase, we also demonstrate in this study that Src could phosphorylate HRas, but most intriguingly, PHLDA1 could inhibit Ras phosphorylated by Src tyrosine kinase. In addition, in vitro kinase assay also confirmed the result. These observations suggest that PHLDA1 promotes Ras activity via inhibiting its phosphorylation by Src tyrosine kinase. Taken together, we demonstrate in this study a new interacted protein of Ras, PHLDA1. Up-regulation of PHLDA1 promoted glioma progression and recurrence. PHLDA1 involved in Ras/Raf/Mek/Erk signaling pathway. Moreover, PHLDA1 and Src competed binding with the same domain of Ras, then promoted Ras activity via inhibiting Ras phosphorylation by Src tyrosine kinase. Our findings may provide a new idea of the molecular mechanism underlying glioma progression and a novel potential therapeutic target for comprehensive glioblastoma treatment.

Materials and methods

Cell culture and reagents

One normal human astrocyte (HEB), HEK293T cells and GBM cell lines (U87, U251 T98G, U118 and LN229) were purchased from Chinese Academy of Sciences. All cells were mycoplasma-free and authenticated by cellular morphology and STR analysis. Cells were cultured at 37 °C in a 5% CO2 humidified incubator and not cultured for more than 2 months. All were maintained in Dulbecco’s modified Eagle’s medium, while T98G was cultured in modified Eagle’s medium with penicillin (100 units/mL), streptomycin (100 µg/mL) and 10% FBS (Biological Industries, Kibbutz Beit-Haemek, Israel). Transfection was performed using Lipofectamine 3000 (L3000001, Invitrogen Life Technologies, Carlsbad, CA, USA) for HEB and GBM cell lines and Simple-Feet Reagent (Signaling Dawn Biotech, Wuhan, Hubei, China) for 293 T cells following the manufacturer’s instructions.

The reagents used were Cobimetinib (# HY-13064, MedChemExpress, USA), Kobe0056 (#T1876, TargetMol, USA). The recombinant protein used were human active Src protein (#10755-H20B, Sinobilogical, China), HRas protein (12059-H08B, Sinobilogical, China). Antibodies against PHLDA1 (# ab133654), Ras (# ab52939) were purchased from Abcam (Cambridge, MA). p-Raf 1(# sc-271928), goat anti-rabbit-HP (sc-2004) and goat anti-mouse-HP (sc-2005) were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Goat anti-rabbit-cy3 (#A10520), Goat anti-rabbit-488 (# F2761), were purchased from Invitrogen. Erk1/2 (#4695), Erk1/2-HRP (#4348), Flag (#14793); HA (#3724), GST (#2624), RSK1/RSK2/RSK3 (# 9355), p-p90RSK (Ser380) (#11989), Rho-GTPase Antibody Sampler Kit (# 9968), Raf (#53745), CREB (#9197), p-CREB (Ser133) (#9198), glyceraldehyde 3-phosphate dehydrogenase (# 2118) were purchased from Cell Signaling Technology (Danvers, MA).

Plasmids construction

The PHLDA1 plasmid was purchased from Youbao Bio-technology Company (Changsha, China), the full length and truncated mutation (PHL and PQ domain) were subcloned into the pcDNA3.1–3×Flag and pCMV-HA-N vector, respectively. HRas (ΔSWI,ΔSWII and ΔSWI&II) domain-deleted mutant fragments were subcloned into the pcDNA3.1–3×Flag vector. The primers used for cloning into these constructs are as follows:

PHL-HA F: CGGAATTCAGCTTATGCTGGGAG GATG
PHL-HA R: GGGGTACCCTACTGACAGATCTTG TACT
PQ-HA F: CGGAATTCAAGTGTCGACCAGCA GCCC
PH-HA R: GGGGTACCATCCAGGAGTTGGAGGT PHL-Flag F: CCCAAGCTATGTCTATGCTGGG AGGA
PHL-Flag R: CCGGAATTCTGACAGATCTTG TACT
PQ-Flag F1: CCGGAATTCAGCTTATGCTGGGAGGAGT
PQ-Flag R: CCGGAATTCTGACAGATCTTG TACT
HRAS ΔSWI F: CATTTTGTTGACCTCCTACCAGGA CAGGT
HRAS ΔSWI R: CTCCCGTGGAG GTCACAAATGTG TCT
HRAS ΔSWII F: TTGGACATCTCG GACCAGTAC ATGCAC
HRAS ΔSWII R: CATGTACTCGTC GACCAGTAC ATGCAC
HRAS ΔSWI&II F: CATTTTGTTGAC GACCAGTAC ATGCAC
HRAS ΔSWI&II R: CATTTTGTTGAC GACCAGTAC ATGCAC

© Springer
PHLDA1 knockdown reduced tumor formation in vivo. A U87-bearing xenograft mouse model was established, representative images were taken on day 28 after cell injected into the mouse brain. U87 cells were a stable expression of green fluorescent protein (GFP) using a vector encoding enhanced GFP (pEGFP-C1). U87-GFP expressing cloned cells were stably infected with lentiviral scramble, shPHLDA1#1 or shPHLDA1#2, subsequently, these GFP-expressing cloned cells were implanted into the brain of severely compromised immunodeficient (SCID) mice, tumor fluorescence was monitored at the beginning time of injection by the IVIS Spectrum In Vivo Imaging System. B Quantification of tumor fluorescence intensity demonstrated a statistically significant reduction in PHLDA1 knockdown mice compared to scramble mice. C Representative photographs of HE staining for each group are shown. D Tumor area was measured from scramble, shPHLDA1#1 or shPHLDA1#2 mice brain slice. E Kaplan–Meier survival curve was plotted for scramble, shPHLDA1#1 or shPHLDA1#2 mice. F Tumors were excised from SCID mice at the end of the experiment. Photographs of tumors from each group are shown. G Tumor volume was measured after tumors were excised, length × width2 × 0.5. H Tumor weight was measured after tumors were excised. I Representative photographs of immunohistochemistry analysis for each antibody and each group are shown. J The integrated optical density (IOD) of PHLDA1, p-raf1 and Ki-67 expressions in xenograft tissues was evaluated using the Image-Pro premier software offline (v9.0) program. *P < 0.05, **P < 0.01, ***P < 0.001. Error bars represent the mean ± SD from three independent experiments.

**Fig. 7** PHLDA1 promotes glioblastoma cell growth via sustaining the activation state of Ras

HRAS ΔSWI&II R: CATGTACTGGTC GTCCACAAA ATGGTTCT

Cell proliferation assay and soft agar colony formation assay

Viable cells were seeded into 96-well plates (1.5–3 × 10^5 cells per well). Cell proliferation was determined by MTT assays, and absorbance was measured at 0, 24, 48, 72 and 96 h after seeding the cells. For the plate colony formation assay, 200 viable cancer cells were triply seeded in six-well plates in 2 mL of cell culture medium. After two weeks, colonies were stained with 0.2% crystal violet and counted under a microscope. For the soft agar colony formation assay, 8000 viable cancer cells were seeded in triplicate in 1 mL of cell culture medium with 2 mM glutamine, 5 μg/mL gentamycin and 0.3% soft agar. The mixture was layered onto 0.5% solidified agar in a cell culture medium in six-well plates. After one to three weeks, colonies were counted and photographed under a microscope using the Image-Pro Plus software (v.6.0) program (Media Cybernetics, Rockville, MD).

Lentiviral construction and infection

The short hairpin RNA (shRNA) targeting PHLDA1 (shPHLDA1#1 and shPHLDA1#2). The sh-PHLDA1 sequences were cloned into the pLKO.1 backbone at the AgeI and EcoRI restriction sites. The pLKO.1-puro nontarget shRNA control plasmid DNA (Scramble) was purchased from Sigma-Aldrich. The shPHLDA1 sequences are designed as follows:

ShPHLDA1#1F: 5′-CCGAGGCGCAAGGGAAGTAC TTGTCTCGAGACATGTACTTGGCCCTTTGCGCTTT GCTTG-3′
ShPHLDA1#1R: 5′-AACCTTTTTCGAGTAATTT CCTATCTCGAGTAAGATTACTACGGATATTGTT GCTTG-3′
ShPHLDA1#2F: 5′-CCGGCTATTCGGTAATT CCTATCTCGAGTAAGATTACTACGGATATTGTT GCTTG-3′
ShPHLDA1#2R: 5′-AATTTTAAAACCTATCCGTA GTACTTCTACTCGAGTAAGATTACTATTAC GGGG-3′

Immunohistochemistry

The human glioma tissue array was purchased from Shanghai Outdo Biotech Co. Ltd (Cat# HBraG177Su01; Shanghai, China). The array was deparaffinized in xylene solution and rehydrated using gradient ethanol concentrations. Antigen retrieval was performed using sodium citrate and the slides were then incubated with H2O2 to block endogenous peroxidases. Thereafter, the primary antibodies PHLDA1 (1:100) was incubated at 4 °C overnight and the signals were visualized by the indirect avidin-biotin-enhanced horseradish peroxidase method according to the manufacturer’s instructions (Vector Laboratories, Burlingame, CA). After development, all sections were observed by microscope (400×) and quantitative analysis was performed using the Image-Pro Premier software (v.9.0) program (Media Cybernetics, Rockville, MD).

Immunofluorescent (IF) labeling

Cells (1 × 10^5) plated on gelatin-coated coverslips were fixed in 4% paraformaldehyde for 1 h, permeabilized with 0.1% Triton X-100, and blocked with 10% goat serum. Antibodies were each applied at a 1:100 dilution. Appropriate fluorescence-conjugated secondary antibodies were used at 1:200 after washing. Coverslips were counterstained with DAPI and mounted with DAKO® fluorescent mounting medium (Dako Corporation, Carpinteria, CA). The stained cells were visualized using a Nikon confocal microscope A1R + and Nis-Elements software. Each image is a single Z section taken at the same cellular level.

Western blotting

Cells were washed with ice-cold PBS twice and disrupted with lysis buffer (20 mM Tris–HCl, pH 7.5, 2 mM EDTA, 100 mM NaCl, 5 mM MgCl2, 1% (v/v) Triton X-100, 5 mM

© Springer
NaF, 10% (v/v) glycerol, 0.5 (v/v) 2-mercaptoethanol, 0.1 mM Na₃VO₄ and protease inhibitors). Cell lysate was centrifuged at 12,000 × g for 5 min at 4 °C and supernatant fractions were collected. Protein extracts (30–50 μg) were separated by SDS-PAGE and transferred to polyvinylidene difluoride membranes (Millipore, Billerica, MA) in 20 mM Tris–HCl (pH 8.0), containing 150 mM glycine and 20% (v/v) methanol. Membranes were blocked with 5% non-fat milk in TBS containing 0.05% Tween-20 (TBS-T) and incubated with their specific primary antibodies 4°C overnight. Blots were washed 3 times in TBS-T buffer, followed by incubation with an appropriate HRP-conjugated secondary antibody for 1 h at room temperature for hybridization. The blots were visualized using the enhanced chemiluminescence (ECL) reagent (Millipore, Billerica, MA).

**Immunoprecipitation**

Cells were washed with ice-cold phosphate-buffered saline and disrupted in lysis buffer (25 mM Tris, 250 mM NaCl, 0.1% Triton X-100, 10% glycerol, 1 mM NaVO₃, 1 mM NaF, protease cocktail inhibitors pH 8.0). The cells were vortexed, sonicated and then centrifuged at 20,000 × g for 30 min at 4 °C. The protein concentrations of the lysates were measured as above and protein concentration was equalized for all samples. The samples were then precleared with rabbit IgG and protein A/G Plus agarose for 1 h with rotation at 4 °C. Finally, the samples were immunoprecipitated with 2.5 µg of PHLDA1 antibody overnight followed by incubation with 20 µL protein A/G agarose beads for another 2 h at 4 °C. The beads were then washed five times and resuspended in 2 × Laemmli sample buffer. The samples were analyzed by SDS-PAGE.

**Mouse in situ GBM model**

This study was approved by the Ethics Committee of Zhengzhou University (Zhengzhou, Henan, China). Chimeric BM mice (male 8 weeks) were anaesthetized using 0.5 mg/g of intraperitoneal injection of 20 mg/ml Avertin (Sigma-Aldrich) and 5 mg/kg of the pre-surgical analgesic Anafen 1 mg/ml (Ketoprofen) was administered subcutaneously.
For in vitro kinase assays, 200 ng his-Ras recombinant protein was first incubated 100 μM unlabeled GTP for 15 min in kinase buffer (20 mmol/L HEPES, pH 7.4, 10 mmol/L MgCl₂, 5 mmol/L EGTA, 150 mmol/L NaCl, 20 mmol/L β-glycerol phosphate), and then incubated with 100 ng GST-Src and 100 μM ATP, with or without 200 ng HA-PHLDA1 purified protein. The reactions were run in kinase buffer for 30 min at 30 °C and stopped by adding 6 × SDS sample buffer. Phosphorylated proteins were separated by SDS/PAGE and analyzed by immunoblotting.

### Ras activity assay

Ras activity was assessed using the Ras activation assay kit from Thermofisher Scientific (#16117). Briefly, Ras-GTP from variously treated lysates was pulled down using the GST fusion protein corresponding to the human Ras-binding domain of Raf-1 bound to agarose. The presence of Ras-GTP was detected by western blotting using anti-Ras antibody.

### Purification of recombinant PHLDA1 proteins

Wild-type PHLDA1 expression constructs were generated by subcloning into the pet28a vector. Plasmids were transformed into Escherichia coli strain BL21 and protein expression was induced at 16 °C overnight by 0.5 mM isopropyl-β-D-thiogalactopyranoside (IPTG). Cells were disrupted and resuspended in lysis buffer (50 mM NaH₂PO₄, 300 mM NaCl, 10 mM imidazole, 1 mM phenylmethanesulfonyl fluoride, 1% Triton X-100). After sonication, the supernatants of the cell lysates were incubated with NIT-NTA agarose (QIAGEN, Germantown, MD, USA). The beads were washed four times with washing buffer (50 mM NaH₂PO₄, 300 mM NaCl, 60 mM imidazole), and purified proteins were eluted with elution buffer (50 mM NaH₂PO₄, 300 mM NaCl, 300 mM imidazole). The purity of the protein was determined by SDS-PAGE followed by Coomassie brilliant blue (CBB) staining.

### Statistical analysis

Statistical analysis was carried out by unpaired Student’s t-test using GraphPad Prism software. For survival data, the Kaplan–Meier method was used to estimate survival fractions and the log-rank test was used to evaluate the difference in survival between different groups. P values were adjusted for multiple comparisons by Holm’s procedure. Each experiment was performed 3 times independently. All quantitative data are expressed as mean values ± standard error of the mean (SD) and significance between groups was determined by one-way ANOVA unless otherwise indicated (*P < 0.05, **P < 0.01 and ***P < 0.001).

### Author contributions

ZD and KL designed and supervised the experiments; JW prepared the manuscript; JW, NY, YH, MW, LY, performed experiments; JW, ML, SP and XL performed data analysis and interpretation.
References

1. Louis DN, Holland EC, Cairncross JG (2001) Glioma classification: a molecular reappraisal. Am J Pathol 159(3):779–786. https://doi.org/10.1016/S0002-9440(10)61750-6

2. Kleihues P, Ohgaki H (1999) Primary and secondary glioblastomas: from concept to clinical diagnosis. Neuro Oncol 1(1):44–51. https://doi.org/10.1038/neo01.1.44

3. Holland EC (2001) Gliomagenesis: genetic alterations and mouse models. Nat Rev Genet 2(2):120–129. https://doi.org/10.1038/35052535

4. Jiang BH, Aoki M, Zheng JZ, Li J, Vogt PK (1999) Myogenic signaling of phosphatidylinositol 3-kinase requires the serine-threonine kinase Akt/protein kinase B. Proc Natl Acad Sci U S A 96(5):2077–2081. https://doi.org/10.1073/pnas.96.5.2077

5. Mattingly RR (2013) Activated ras as a therapeutic target: constraints on directly targeting ras isoforms and wild-type versus mutated proteins. ISRN Oncol 2013:536529. https://doi.org/10.1155/2013/536529

6. DeClue JE, Papageorge AG, Fletcher JA, Diehl SR, Ratner N, Vass WC, Lowy DR (1992) Abnormal regulation of mammalian p21ras contributes to malignant tumor growth in von Recklinghausen (type 1) neurofibromatosis. Cell 69(2):265–273. https://doi.org/10.1016/0092-8674(92)90407-4

7. Haslam RJ, Koide HB, Hemmings BA (1993) Pleckstrin domain homology. Nature 363(6427):309–310. https://doi.org/10.1038/363309b0

8. Ingley E, Hemmings BA (1994) Pleckstrin homology (PH) domains in signal transduction. J Cell Biochem 56(4):436–443. https://doi.org/10.1002/jcb.240560403

9. Saraste M, Hyvonen M (1995) Pleckstrin homology domains: a fact file. Curr Opin Struct Biol 5(3):403–408. https://doi.org/10.1016/0959-440x(95)80104-9

10. Nagai MA, Fregnani JH, Netto MM, Brentani MM, Soares FA (2007) Down-regulation of PHLDA1 gene expression is associated with breast cancer progression. Breast Cancer Res Treat 106(1):49–56. https://doi.org/10.1007/s10549-006-9475-6

11. Neef R, Kuske MA, Prols E, Johnson JP (2002) Identification of the human PHLDA1/TDAG51 gene: down-regulation in metastatic melanoma contributes to apoptosis resistance and growth deregulation. Cancer Res 62(20):5920–5929
26. Simanshu DK, Nissley DV, McCormick F (2017) RAS proteins and their regulators in human disease. Cell 170(1):17–33. https://doi.org/10.1016/j.cell.2017.06.009

27. Iversen L, Tu HL, Lin WC, Christensen SM, Abel SM, Iwig J, Wu HJ, Gureasko J, Rhodes C, Petit RS, Hansen SD, Thill P, Yu CH, Stamou D, Chakraborty AK, Kuriyan J, Groves JT (2014) Molecular kinetics. Ras activation by SOS: allosteric regulation by altered fluctuation dynamics. Science 345(6192):50–54. https://doi.org/10.1126/science.1250373

28. Hai J, Liu S, Bufe L, Do K, Chen T, Wang X, Ng C, Li S, Tsao MS, Shapiro GI, Wong KK (2017) RAS proteins and their regulators in human disease. Cell 170(1):17–33. https://doi.org/10.1016/j.cell.2017.06.009

29. Hai J, Liu S, Bufe L, Do K, Chen T, Wang X, Ng C, Li S, Tsao MS, Shapiro GI, Wong KK (2017) RAS proteins and their regulators in human disease. Cell 170(1):17–33. https://doi.org/10.1016/j.cell.2017.06.009

30. Johnson EO, Chang KH, de Pablo Y, Ghosh S, Mehta R, Badve S, Shah K (2011) PHLDA1 is a crucial negative regulator and effector of Aurora A kinase in breast cancer. J Cell Sci 124(Pt 16):2711–2722. https://doi.org/10.1242/jcs.084970

31. Li G, Wang X, Hibshoosh H, Jin C, Halmos B (2014) Modulation of ErbB2 blockade in ErbB2-positive cancers: the role of ErbB2 Mutations and PHLDA1. PLoS ONE 9(9):e106349. https://doi.org/10.1371/journal.pone.0106349

32. Coutinho-Camillo CM, Lourenco SV, Nonogaki S, Vartanian JG, Nagai MA, Kowalski LP, Soares FA (2013) Expression of PAR-4 and PHLDA1 is prognostic for overall and disease-free survival in oral squamous cell carcinomas. Virchows Arch 463(1):31–39. https://doi.org/10.1007/s00428-013-1438-9

33. Chiu ST, Hsieh FJ, Chen SW, Chen CL, Shu HF, Li H (2005) Clinicopathologic correlation of up-regulated genes identified using cDNA microarray and real-time reverse transcription-PCR in human colorectal cancer. Cancer Epidemiol Biomarkers Prev 14(2):437–443. https://doi.org/10.1158/1055-9965.EPI-04-0396

34. Ren L, Mendoza A, Zhu J, Briggs JW, Halsey C, Hong ES, Burkett SS, Morrow J, Lizardo MM, Osborne T, Lu SQ, Luu HH, Meltzer P, Khanna C (2015) Characterization of the metastatic phenotype of a panel of established osteosarcoma cells. Oncotarget 6(30):29469–29481. https://doi.org/10.18632/oncotarget.5177

35. Liu L, Shi Y, Shi J, Wang H, Sheng Y, Jiang Q, Chen H, Li X, Dong J (2019) The long non-coding RNA SNHG1 promotes glioma progression by competitively binding to miR-194 to regulate PHLDA1 expression. Cell Death Dis 10(6):463. https://doi.org/10.1038/s41419-019-1698-7

36. Buday L, Vas V (2020) Novel regulation of Ras proteins by direct tyrosine phosphorylation and dephosphorylation. Cancer Metastasis Rev 39(4):1067–1073. https://doi.org/10.1007/s10555-020-09918-2

37. Buday L, Downward J (2008) Many faces of Ras activation. Biochim Biophys Acta 1786(2):178–187. https://doi.org/10.1016/j.bbcan.2008.05.001

Publisher's Note Springer Nature remains neutral with regard to jurisdictional claims in published maps and institutional affiliations.