PHLDB2 Mediates Cetuximab Resistance via Interacting With EGFR in Latent Metastasis of Colorectal Cancer

Maochao Luo,1,2,* Zhao Huang,1,2,* Xingyue Yang,1,2,* Yan Chen,1,2,* Jingwen Jiang,1,2 Lu Zhang,1,2 Li Zhou,1,2 Siyuan Qin,1,2 Ping Jin,1,2 Shuyue Fu,1,2 Liyuan Peng,1,2 Bowen Li,1,2 Yongting Fang,1,2 Wenchen Pu,1 Yanqi Gong,1 Yu Liu,1 Zhixiang Ren,1 Qiu-Luo Liu,3 Cun Wang,3 Fangqiong Xiao,2 Du He,4 Hongying Zhang,4 Changlong Li,2 Heng Xu,1 Lunzhi Dai,1 Yong Peng,1 Zong-Gung Zhou,3 Canhua Huang,1,2 and Hai-Ning Chen3

1State Key Laboratory of Biotherapy and Cancer Center, West China Hospital, Sichuan University, and Collaborative Innovation Center for Biotherapy, Chengdu, China; 2West China School of Basic Medical Sciences & Forensic Medicine, Sichuan University, Chengdu, China; 3Department of Gastrointestinal Surgery, State Key Laboratory of Biotherapy and Cancer Center, West China Hospital, Sichuan University, Chengdu, China; and 4Department of Pathology, West China Hospital, Sichuan University, Chengdu, China

SUMMARY

Pleckstrin homology-like domain, family B, member 2 (PHLDB2) is up-regulated by chemotherapeutic agent-induced oxidative stress in latent liver metastasis of colorectal cancer. Moreover, up-regulated PHLDB2 stabilizes epidermal growth factor receptor and promotes its nuclear translocation, resulting in epidermal growth factor receptor signaling activation and cetuximab resistance.

BACKGROUND & AIMS: Latent metastasis of colorectal cancer (CRC) frequently develops months or years after primary surgery, followed by adjuvant therapies, and may progress rapidly even with targeted therapy administered, but the underlying mechanism remains unclear. Here, we aim to explore the molecular basis for the aggressive behavior of latent metastasis in CRC.

METHODS: Transcriptional profiling and pathway enrichment analysis of paired primary and metastatic tumor samples were performed. The underlying mechanisms of pleckstrin homology-like domain, family B, member 2 (PHLDB2) in CRC were investigated by RNA immunoprecipitation assay, immunohistochemistry, mass spectrometry analysis, and Duolink in situ proximity ligation assay (Sigma-Aldrich, Shanghai, China). The efficacy of targeting PHLDB2 in cetuximab treatment was elucidated in CRC cell lines and mouse models.

RESULTS: Based on the transcriptional profile of paired primary and metastatic tumor samples, we identified PHLDB2 as a potential regulator in latent liver metastasis. A detailed mechanistic study showed that chemotherapeutic agent-induced oxidative stress promotes methyltransferase-like 14 (METTL14)-mediated N6-methyladenosine modification of PHLDB2 messenger RNA, facilitating its protein expression. Up-regulated PHLDB2 stabilizes epidermal growth factor receptor (EGFR) and promotes its nuclear translocation, which in turn results in EGFR signaling activation and consequent cetuximab resistance. Moreover, Arg1163 (R1163) of PHLDB2 is crucial for interaction with EGFR, and the R1163A mutation abrogates its regulatory function in EGFR signaling.

CONCLUSIONS: PHLDB2 plays a crucial role in cetuximab resistance and is proposed to be a potential target for the treatment of CRC. (Cell Mol Gastroenterol Hepatol 2022;13:1223–1242; https://doi.org/10.1016/j.jcmgh.2021.12.011)
Colorectal cancer (CRC) is one of the most common cancers and the leading cause of cancer-related death worldwide. Although the clinical outcome of CRC patients has improved with early screening and multidisciplinary therapy, metastasis is still the direct cause of death in most CRC patients and frequently develops months or years after treatment for locoregional CRC. Latent metastasis is a major hurdle in CRC treatment because these latency competent cancer cells are adaptively resistant to chemotherapy and targeted therapy, retain tumor-initiating potential, and can evolve into systemic metastasis rapidly. It has been determined that latent metastasis in patients who already have been treated with chemotherapy is associated with lower response rates, but the underlying mechanism has remained elusive.

A minority of disseminated cancer cells may survive months or years under selective pressure of chemotherapeutic drugs, function as latent seeds, and contribute to metastasis. This is of even greater importance given recent evidence supporting early metastatic dissemination in CRC. Latent metastatic cells may acquire survival-related traits as a result of genomic alteration and epigenetic regulation, which provides the rationale behind the comparable analysis of paired primary and latent tumor samples in a longitudinal setting. Because the driver gene heterogeneity of metastasis is present only in limited cases, most metastatic lesions are indeed regulated at the transcriptional and post-transcriptional levels. As the most prevalent and abundant post-transcriptional RNA modification in eukaryotic messenger, N6-methyladenosine (m6A) has been shown to play an essential role in human diseases, but whether m6A modification contributes to latent metastasis in CRC is largely unknown.

Cetuximab is a Food and Drug Administration-approved anti–epidermal growth factor receptor (EGFR) monoclonal antibody recommended for metastatic CRC patients with wild-type KRAS/NRAS/BRAF tumors, but its efficacy remains unsatisfactory, especially in patients with latent metastasis who experience progression on adjuvant therapies, which cannot be readily explained by genetic mechanisms. To date, dysregulation of EGFR endocytic trafficking has been linked to the pathogenesis of cancers, but our understanding remains incomplete. Thus, it is of paramount importance to elucidate the underlying mechanism of cetuximab resistance in the latent metastasis of CRC.

To unearth the molecular mechanism of latent metastasis, we performed RNA sequencing and transcriptional profiling in tissues from CRC patients and found that the expression of pleckstrin homology-like domain, family B, member 2 (PHLDB2) is up-regulated significantly in latent metastasis of CRC tissues and highly correlated with poor prognosis. Mechanistically, chemotherapeutic drugs, including 5-fluorouracil (5-FU) and oxaliplatin (OXA), induce the expression of PHLDB2 by promoting m6A modification of PHLDB2 messenger RNA (mRNA). Further analysis showed that PHLDB2 binds and stabilizes EGFR through the Arg1163 site, facilitating EGFR nuclear translocation, thus conferring cetuximab resistance with therapeutic implications.

**Results**

**Identification of PHLDB2 as a Novel Regulator of CRC Progression**

To explore the molecular basis for the aggressive behavior of latent metastasis in CRC, latent liver metastasis (LL) that occurred at least 6 months after primary surgery in conjunction with treatment-naive primary tumors (P) was collected from 5 patients who received postoperative adjuvant treatment, such as capecitabine plus oxaliplatin or 5-fluorouracil, leucovorin, oxaliplatin, while synchronous liver metastasis (SL) with paired primary tumors from 5 CRC patients who underwent simultaneous resection directly with no neoadjuvant treatment received also was obtained as a reference (Figure 1A, Supplementary Table 1). To identify potential regulators associated with latent CRC progression, we performed a transcriptomic analysis of paired primary and metastatic tumor tissues from CRC patients. Comparative genome-wide expression analysis yielded a list of genes that were differentially expressed among synchronous or latent liver metastases and their matched primary tumors (ie, SL vs P, SL/ P; LL vs P, LL/P, and LL vs SL, LL/SL) using an interaction term between patient group (synchronous or latent) and sample type (primary tumor or liver metastasis) (Figure 1B).

Based on pathway enrichment analysis, we found concurrent pathways such as cholesterol metabolism enriched in liver metastasis, possibly owing to metabolic reprogramming in liver colonization (Figure 1C), which is consistent with previous studies. On the other hand, latent liver metastasis was characterized by enriched signaling pathways, including the phosphatidylinositol 3-kinase protein kinase B (PI3K-AKT) signaling pathway and EGFR tyrosine kinase inhibitor resistance, supporting the rapid progressive course of latent metastasis in response to adjuvant treatment, including EGFR-targeted therapy (Figure 1A).

Among the genes that were differentially expressed in both LL/SL and LL/P, PHLDDB2 was the most significant gene (log fold change, >2; adjusted \(P < 1 \times 10^{-7}\)) (Figure 1D, 1E, 1F).
Figure 1. Identification of PHLDB2 as a novel regulator of CRC progression. (A) Clinical course. Latent liver metastasis (LL) that occurred after primary surgery in conjunction with treatment-naïve primary tumors (P) were collected from 5 patients who received postoperative adjuvant treatment, such as CAPEOX or FOLFOX, while synchronous liver metastasis (SL) with paired primary tumors from 5 CRC patients who underwent simultaneous resection directly with no neoadjuvant treatment also were obtained as a reference. Numbers on the timeline indicate months relative to the primary surgery. (B) Heatmap representation of a list of genes differentially expressed among P, SL, and LL (adjusted $P < .005$, significance was determined using DESeq2 analysis using an interaction term). (C) KEGG analysis representing signaling pathways enriched in SL vs P (LL/P), LL vs P (SL/P), and LL vs SL (LL/SL). (D) Identification of PHLDB2 as the most significant gene that was increased significantly in latent liver metastasis compared with primary tumor and synchronous liver metastasis (log fold change, $>2$; adjusted $P < 1 \times 10^{-7}$). (E) Relative mRNA levels of PHLDB2 in paired P, SL, and LL. Left: The relative protein level of PHLDB2 in P, SL, and LL. Right: Representative image of immunohistochemical staining of PHLDB2 in P, SL, and LL. Scale bars: 50 μm. (G) Kaplan–Meier curves representing the proportion of progression-free or overall survival stratified according to PHLDB2 mRNA levels in CRC patients from 3 independent cohorts (TCGA, GSE17536, and GSE28722). Survival analysis was performed using the log-rank test in panel G. **$P < .01$, ***$P < .001$. Akt, protein kinase B; CAPEOX, capecitabine plus oxaliplatin; CTX, cetuximab; ECM, extracellular matrix; FOLFIRI, folinic acid, 5-fluorouracil and irinotecan; FOLFOX, 5-fluorouracil, leucovorin, oxaliplatin; MAPK, mitogen-activated protein kinase; PI3K, phosphatidylinositol 3-kinase; Ras, Ras GTPase; Rap1, Rap1 GTPase; TPM, transcripts per million.
Supplementary Table 2), whose mRNA expression was increased significantly in latent liver metastasis compared with primary tumor and synchronous liver metastasis (Figure 1F). Consistently, the protein levels of PHLDB2 also were up-regulated in latent liver metastasis, as evidenced by immunohistochemical (IHC) staining (Figure 1F). To further address the association between PHLDB2 and CRC progression, we assessed the prognosis of CRC patients in relation to the mRNA level of PHLDB2 in 3 independent cohorts (ie, The Cancer Genome Atlas, GSE17536, and GSE28722). The results indicated that high expression of PHLDB2 confers poor overall and progression-free survival (Figure 1G). Taken together, these results suggest a potential role of PHLDB2 in latent CRC progression.

Oxidative Stress-Induced m6A Modification Promotes the Expression of PHLDB2

To investigate the mechanism underlying the up-regulation of PHLDB2 in the latent metastasis of CRC patients, we examined both the protein and mRNA levels of PHLDB2 after administration of 5-FU or OXA in 3 CRC cell lines (ie, SW480, DLD-1, and NCI-H508). Consistently higher levels of PHLDB2 were observed after treatment with 5-FU or OXA (Figure 2A and B). Because the generation of reactive oxygen species (ROS) is a common effect of 5-FU and OXA (Figure 2C), we determined whether the ROS scavenger N-acetyl-L-cysteine may reverse this phenomenon. To this end, we found that N-acetyl-L-cysteine attenuated 5-FU-induced or OXA-induced PHLDB2 expression (Figure 2A and B). Furthermore, Western blot analysis showed that when treated with hydrogen peroxide (H2O2) to mimic oxidative stress, PHLDB2 was increased in a time-dependent manner (Figure 2D), which also was confirmed by quantitative reverse-transcription polymerase chain reaction (qRT-PCR) (Figure 2E and F). These results support that overproduction of ROS is responsible for the chemotherapeutic drug 5-FU- or OXA-induced PHLDB2.

Next, we explored the mechanism by which ROS regulate the expression of PHLDB2. In the presence of the transcription inhibitor actinomycin D, the PHLDB2 mRNA degradation rate was evaluated using qRT-PCR for the indicated times. We found that H2O2 reduced the degradation of PHLDB2 mRNA (Figure 2G), which suggests that treatment with H2O2 may facilitate the expression of PHLDB2 by improving its mRNA stability. Because m6A modification is one of the most prevalent modifications in eukaryotic mRNAs and is highly associated with mRNA stability, we investigated the level of m6A modification in PHLDB2 mRNA and found that it was increased significantly in CRC cells treated with H2O2 (Figure 2H). To determine the underlying mechanism, we analyzed the correlation between PHLDB2 and m6A writers in CRC using TCGA data. Notably, we found that the m6A writer methyltransferase-like 14 (METTL14) was associated significantly with PHLDB2 mRNA levels (R = 0.34; P < .001, Pearson correlation analyses) (Figure 2I and J), which was validated further by Western blot assays (Figure 2K). To confirm whether METTL14 is required for H2O2-induced high expression of PHLDB2, we knocked down METTL14 in NCI-H508 cells and found that loss of METTL14 repressed H2O2-induced expression of PHLDB2 at both the protein and mRNA levels (Figure 2L and M). Collectively, these data suggested that up-regulated PHLDB2 in latent metastatic tumor samples may be attributed to METTL14-mediated m6A modification of PHLDB2 mRNA in response to chemotherapeutic treatment.

PHLDB2 Enhances Cetuximab Resistance and Metastatic Potential In Vitro and In Vivo

To ascertain the function of PHLDB2, we constructed DLD-1 and NCI-H508 cells stably overexpressing PHLDB2, and SW480 cells stably knocked down PHLDB2. We found that knockdown of PHLDB2 significantly reduced the levels of EGFR and the phosphorylation levels of EGFR downstream signaling molecules. In contrast, ectopic expression of PHLDB2 led to the activation of EGFR signaling (Figure 3A). The 3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2H-tetrazolium bromide (MTT) assay and colony formation assay were performed, showing that PHLDB2 had no significant effect on CRC cell proliferation (Figure 3B and C). We next evaluated the effects of PHLDB2 on the metastatic potential of CRC cells and found that migration and invasion were enhanced in PHLDB2-overexpressing cells (Figure 3D). In contrast, knockdown of PHLDB2 showed opposite effects (Figure 3E). The regulation of metastasis by PHLDB2 in vivo was determined further: DLD-1-Vector or -PHLDB2 cells were injected into the spleen of BALB/c nude mice, and liver metastatic lesions were analyzed by H&E staining. Consistent with the in vitro results, overexpression of PHLDB2 promoted the metastatic capacity of CRC cells (Figure 3F).

Given the aggressive nature of latent metastasis in response to adjuvant therapy, including EGFR-targeted therapy, we next analyzed the mRNA expression of PHLDB2 in 19 CRC cells using the Cancer Cell Line Encyclopedia database in combination with their cetuximab sensitivity based on published reports. As shown in Figure 4A, a negative correlation was observed between PHLDB2 expression and cetuximab efficacy (r = −0.52; P = .023, Pearson correlation). As such, we hypothesized that PHLDB2 may contribute to cetuximab resistance in CRC cells. To confirm the impact of PHLDB2 on cetuximab efficacy, MTT assays, colony formation assays, and soft agar colony formation assays were performed, showing that PHLDB2 knockdown sensitized SW480 cells to cetuximab, whereas the exogenous expression of PHLDB2 led to a reduction in the cetuximab response rate in DLD-1 and NCI-H508 cells (Figure 4B–E).

To determine whether PHLDB2 promotes cetuximab resistance by regulating EGFR signaling, the activation of EGFR signaling was evaluated in PHLDB2 overexpression or knockdown cells with or without cetuximab treatment. In the absence of cetuximab, overexpression of PHLDB2 significantly up-regulated the expression of EGFR and the phosphorylation of EGFR downstream molecules, including Akt and extracellular signal–regulated kinase, suggesting activation of EGFR signaling (Figure 4F and G). In the presence of cetuximab, EGFR signaling was inhibited in PHLDB2-low cells (NCI-H508-vector and SW480-shPHLDB2), but remained unchanged in PHLDB2-high
Figure 2. Oxidative stress-induced m^6^A modification promotes the expression of PHLDB2. (A) Protein expression of PHLDB2 was measured by immunoblotting after treatment with 5-FU (2.5 μmol/L) or OXA (5 μmol/L) with or without N-acetyl cysteine (NAC, 2.5 mmol/L) for 24 hours. (B) qRT-PCR assays showing the mRNA level of PHLDB2 in the treatment same as Figure 2A. (C) The level of ROS in CRC cells was measured by flow cytometry after treatment with 5-FU (2.5 μmol/L) or OXA (5 μmol/L) with or without NAC (2.5 mmol/L) for 24 hours. (D) The protein level of PHLDB2 was determined by Western blot after treatment with 0.1 mmol/L H_2O_2 for the indicated times. (E) The mRNA level of PHLDB2 was determined by qRT-PCR after treatment with 0.1 mmol/L H_2O_2 for 24 hours. (F) The level of ROS in CRC cells was measured by flow cytometry after treatment of 0.1 mmol/L H_2O_2 for the indicated time. (G) qRT-PCR assays showing the mRNA degradation rate of PHLDB2 after treatment with actinomycin D (Act D, 2 μg/mL) with or without 0.1 mmol/L H_2O_2 for the indicated time. (H) Methylated RNA immunoprecipitation-qPCR (MeRIP-qPCR) analysis was performed to show H_2O_2-mediated PHLDB2 m^6^A modifications. (I) The correlation between PHLDB2 and mRNA expression of m^6^A writers was analyzed in CRC using TCGA data. (J) The correlation between PHLDB2 and METTL14 mRNA expression was analyzed in CRC using TCGA data (r = 0.34; P < .001, Pearson correlation). (K) Protein levels of METTL3 and METTL14 were determined by immunoblotting after treatment with 0.1 mmol/L H_2O_2 for the indicated times. (L and M) The protein and mRNA levels of PHLDB2 were detected by Western blot or qRT-PCR with or without H_2O_2 treatment (0.1 mmol/L, 24 hours) in CRC cells transfected with siRNA targeting METTL14. *P < .05, **P < .01, ***P < .001. Ctr, Control; siNC, small interfering negative control; TPM, transcripts per million.
These results indicate that PHLDB2 is sufficient to activate EGFR signaling, and this function overcomes the inhibitory effect of cetuximab on EGFR, thus conferring cetuximab resistance and enhancing metastatic potential in CRC cells. Meanwhile, we evaluated the in vivo impact of PHLDB2 on cetuximab resistance of CRC in BALB/c nude mice in which NCI-H508-Vector or -PHLDB2 cells were injected.
Figure 4. PHLDB2 promotes cetuximab resistance in vitro and in vivo. (A) The correlation between the PHLDB2 mRNA level and cetuximab inhibition rate was analyzed in CRC cell lines using Cancer Cell Line Encyclopedia data. (B) The inhibition effect of cetuximab (25 μg/mL) was detected by soft agar colony formation assays (3 weeks). Scale bars: 100 μm. (C–E) The inhibition rate of cetuximab was detected by MTT (48 hours) or colony formation assays (1 wk). (F and G) Western blot analysis showing the effects of PHLDB2 on EGFR downstream signaling in (F) NCI-H508 and (G) SW480 cells treated with cetuximab (25 μg/mL) for the indicated times. (H) A subcutaneous xenograft model was established in BALB/c nude mice injected with NCI-H508 vector and NCI-H508 PHLDB2 cells. One week after injection, the diameters of tumors were measured every 3 days. Twenty-one days later, the mice were killed, and the tumors were dissected and photographed. (I and J) Tumor xenografts in panel H were evaluated for the proliferation markers Ki67 and cleaved caspase 3 by immunohistochemistry. Scale bars: 50 μm. (K) Representative immunohistochemical images showing the PHLDB2 protein levels in human CRC cases treated with cetuximab. *P < .05, **P < .01, ***P < .001. P values were calculated as follows: (A) Pearson correlation, (B–E, and H–J) Student t test, or (K) chi-square test, and data are presented as the means ± SD from at least 3 independent experiments. CTX,
subcutaneously in the right flank region. Upon treatment with cetuximab, the anticancer efficacy was decreased significantly in the PHLDB2-overexpression group compared with the vector group (Figure 4H). Moreover, overexpression of PHLDB2 led to increased cell proliferation and decreased apoptosis in the presence of cetuximab, as evidenced by IHC staining analysis (Figure 4I and J). To further explore the correlation between PHLDB2 and cetuximab response, we collected tumor samples from 15 CRC patients administered cetuximab, including 6 progressive disease cases, 6 stable disease cases, and 3 partial response cases. Consistently, patients with higher PHLDB2 expression were enriched significantly in the progressive disease group (Figure 4K). Taken together, these results underscore a potential causal role of PHLDB2 in promoting cetuximab resistance and metastasis of CRC cells.

**PHLDB2 Protects EGFR From Ubiquitin-Mediated Degradation and Mediates EGFR Nuclear Localization**

To understand how PHLDB2 regulates EGFR expression, the protein synthesis inhibitor cycloheximide and proteasome inhibitor MG132 were used to inhibit protein synthesis or block proteasome-mediated protein degradation, respectively. In PHLDB2 knockdown cells, EGFR underwent rapid degradation upon cycloheximide treatment (Figure 5A), whereas overexpression of PHLDB2 was shown to stabilize EGFR protein (Figure 5B). Consistently, administration of MG132 restored the protein level of EGFR in PHLDB2-low cells (Figure 5C and D), suggesting that PHLDB2 protects EGFR from proteasome-mediated degradation. In addition, PHLDB2 resulted in a significant decrease in ubiquitin (Figure 5E) and the E3 ligase Casitas B-lineage lymphoma (c-Cbl) (Figure 5F) conjugated to EGFR, suggesting that PHLDB2 competes with EGFR, dissociates it from its E3 ligase c-Cbl, and thus protects it from degradation. Overall, these data showed that PHLDB2 interacts with EGFR and protects it from ubiquitin-mediated degradation.

Given that cetuximab targets EGFR only in the cell membrane, we postulated that PHLDB2 induces cetuximab resistance by promoting EGFR internalization. Therefore, we determined the localization of EGFR in cells with or without overexpression of PHLDB2. Immunofluorescence assays showed significant enrichment of EGFR in the nucleus in PHLDB2-overexpressing cells (Figure 5G and H). Moreover, the nuclear localization of EGFR was validated further by a nuclear–cytoplasmic fractionation assay (Figure 5I and J). To explore the molecular mechanism by which PHLDB2 promotes the nuclear translocation of EGFR, we detected the expression of Rab11A, a well-known EGFR recycling protein involved in EGFR recycling back to the plasma membrane,24 and found that PHLDB2 reduced the binding affinity between Rab11A and EGFR (Figure 5K). In addition, overexpression of PHLDB2 enhanced the activation of Src kinase (Src) (Figure 5L), which has been reported to mediate the nuclear translocation of EGFR.25 To further confirm the phenotype by which PHLDB2 promotes EGFR nuclear translocation, we determined the expression of the nuclear EGFR downstream target genes Myb-related protein B (B-myb), Aurora A kinase (AURKA), Aurora B kinase (AURKB), and cyclin D1. As shown in Figure 5M, qRT-PCR analysis showed that PHLDB2 promotes nuclear EGFR-mediated transcription, as evidenced by the increased mRNA levels of nuclear EGFR downstream target genes. Overall, these data confirmed that PHLDB2 facilitates EGFR nuclear localization, which might contribute to cetuximab resistance.

**Arg1163 Is Required for the Interaction of PHLDB2 With EGFR**

To elucidate the mechanism underlying PHLDB2-mediated EGFR nuclear localization, PHLDB2 interactors were coimmunoprecipitated (Figure 6A) and subjected to rounds of liquid chromatography and high-throughput mass spectrometry (LC-MS/MS). Then, the identified binding partners overlapped with the structure-based protein–protein interaction database,26 suggesting EGFR as the top hit among the 4 potential PHLDB2 interactors (Figure 6B, Supplementary Tables 3 and 4). The interaction of PHLDB2 with EGFR was confirmed further by coimmunoprecipitation (co-IP) (Figure 6C and D) and proximity ligation assays (Figure 6E). To investigate the molecular basis for the interaction between PHLDB2 and EGFR, we first produced a series of Flag-tagged PHLDB2 truncated mutants according to the literature27 (Figure 6F). In HEK293T cells, each truncated mutant reached a similar and reproducible intensity of protein. Co-immunoprecipitated green fluorescent protein (GFP)-tagged EGFR was observed only in fragments containing the Pleckstrin homology domain (ie, full-length, Δ1-305, Δ306-562, and Δ563-916), indicating that the PH domain of PHLDB2 is required for binding to EGFR (Figure 6G). To further identify the binding site mediating the interaction, molecular docking with ZDOCK calculations was conducted to make a prediction. As a result, we found that the Arg1163 site of PHLDB2, which is localized in the PH domain, may be responsible for its binding to EGFR (Figure 6H). This prediction was validated further by co-IP assays in cells transfected with wild-type (WT) or R1163A mutant PHLDB2, in which the R1163A mutation significantly abolished the binding of PHLDB2 to EGFR (Figure 6I). Moreover, the R1163A-mediated binding behavior in CRC cells also was confirmed by Duolink (Sigma-Aldrich, Shanghai, China) in situ proximity ligation assays (Figure 6J).

In addition, we also investigated the binding sites of EGFR based on molecular docking modeling, which...
suggested that the H988 and S991 residues of EGFR mediate the interaction with PHLDB2 (Figure 6H). To confirm this, Flag-tagged WT, H988A mutated, S991A mutated, and H988A/S991A double-mutated EGFR were tested for the interaction with PHLDB2. Co-IP assays showed that S991A and H988A/S991A double-mutated EGFR significantly abolished the binding of EGFR to PHLDB2 (Figure 6K), indicating that S991 of EGFR is required for the interaction with PHLDB2. Together, these data showed that R1163 of PHLDB2 and S991 of EGFR mediate their interaction.
**R1163A Mutation Diminishes PHLDB2-Mediated Cetuximab Resistance Both In Vitro and In Vivo**

To determine whether the R1163A-mediated interaction with EGFR confers the biological function of PHLDB2, we first evaluated the effect of the R1163A mutation on EGFR signaling, and found that R1163A mutation significantly reduced the levels of EGFR and the phosphorylation levels of EGFR downstream signaling molecules compared with ectopic expression of WT PHLDB2 (Figure 7A). Notably, the R1163A mutation also attenuated the PHLDB2-enhanced metastatic potential of CRC cells (Figure 7D and E), but neither WT PHLDB2 nor R1163A-mutant PHLDB2 affected cell proliferation (Figure 7B and C). On the other hand, R1163A-mutated PHLDB2 failed to activate EGFR signaling regardless of the presence of cetuximab (Figure 8A and B). In addition, mutation of R1163 abrogated the function of PHLDB2 to promote the nuclear localization of EGFR (Figure 8C). Consistently, R1163A-mutated PHLDB2 no longer conferred a phenotype resistant to cetuximab in CRC cells compared with WT PHLDB2 (Figure 8D–G).

To determine the effects of the PHLDB2 R1163A mutation in vivo, a xenograft mouse model was used to evaluate the cetuximab resistance rate. Consistent with the in vitro findings, overexpression of WT PHLDB2 conferred cetuximab resistance, whereas the R1163A mutant failed to induce obvious cetuximab resistance compared with the vector group (Figure 8H). In addition, overexpression of PHLDB2 with mutated R1163A led to increased apoptosis and decreased cell proliferation in response to cetuximab compared with overexpression of WT PHLDB2, as evidenced by IHC staining analysis (Figure 8A and J). Taken together, these data supported that Arg1163 of PHLDB2 mediated its function in EGFR signaling and cetuximab resistance both in vitro and in vivo.

**Discussion**

The clinical observation that metastases may develop after months or years on adjuvant therapy underscores the necessity to evaluate the efficacy of adjuvant therapy on the development of latent metastases. In the present work, we describe a mechanism involved in latent metastasis of CRC. Based on the transcriptional profiling of paired primary tumors and synchronous or metachronous metastases, we identified PHLDB2 as a potential regulator of latent metastasis that is up-regulated in response to adjuvant treatment. Our further work uncovered a role for PHLDB2 in regulating EGFR signaling pathways, thereby facilitating cetuximab resistance and tumor progression.

**PHLDB2** (pleckstrin homology-like domain, family B, member 2), a 160-kilodalton protein that contains a PH domain and 2 coiled-coil domains, has been shown to regulate the migration and invasion of cancer cells. Here, we show that PHLDB2 is up-regulated significantly in latent metastasis of CRC, and increased PHLDB2 levels are associated with poor survival outcomes, possibly owing to aggressive behavior and treatment resistance. Mechanistically, chemotherapeutic agent-induced ROS promote m6A modification of PHLDB2 mRNA. As one of the most prevalent and abundant post-transcriptional RNA modifications in mRNA, m6A modification appears to be an early adaptive mechanism by which cancer cells respond to chemotherapeutic agent-induced oxidative stress. Together, our data show that chemotherapeutic agent-induced ROS promote the expression of PHLDB2 via m6A modification, resulting in latent metastasis.

As a key driver of CRC tumorigenesis, the aberrant activity of EGFR in the initiation and progression of CRC underscores the importance of characterizing its regulatory network. Known as a prototypic receptor tyrosine kinase, EGFR endocytic trafficking also has been implicated in the regulation of EGFR activation, including recycling and degradation. In this study, we identified that PHLDB2 binds to the Ser991 site of EGFR, resulting in a significant decrease in ubiquitin and the E3 ligase c-Cbl conjugated to EGFR, thus suppressing ubiquitin-mediated degradation of EGFR. This is consistent with previous quantitative mass spectrometric analyses showing the association between Ser991 and ubiquitin-mediated degradation of EGFR, in which the S991A mutation decreases conjugated ubiquitin on EGFR. The activation of EGFR and its downstream signaling pathways ultimately may promote CRC cell proliferation and migration, thus making it one of the most important molecular targets in CRC treatment.

Cetuximab is a Food and Drug Administration–approved EGFR monoclonal antibody that binds to the extracellular domain of EGFR and promotes receptor internalization and degradation. Although cetuximab currently is...
Figure 6. Arg1163 is required for the interaction of PHLDB2 with EGFR. (A) Total lysates from HEK293T cells with or without ectopic expression of Flag-tagged PHLDB2 were co-immunoprecipitated with anti-Flag affinity agarose, followed by sodium dodecyl sulfate–polyacrylamide gel electrophoresis and Coomassie brilliant blue staining. (B) The overlap between the protein–protein interactome and LC–MS/MS data show the potential interaction partners of PHLDB2. (C and D) Co-IP assays indicate the interaction between PHLDB2 and EGFR in CRC cells. (E) Proximity ligation assay (PLA) showing the interaction between PHLDB2 and EGFR in NCI-H508 cells. Scale bar: 10 μm. (F) Schematic diagram of PHLDB2 and the truncated PHLDB2 fragments. The numbers represent amino acid residues. (G) Co-IP assay showing the interaction between EGFR and PHLDB2 truncations in HEK293T cells. (H) Molecular docking of 3-dimensional structures predicts the binding of PHLDB2 (red) with EGFR (gray). (I and J) The interaction between EGFR and WT or R1163A-mutated PHLDB2 was determined by (I) co-IP assays and (J) PLA assays. (K) Co-IP assays were performed to evaluate the interaction between PHLDB2 and WT or mutated EGFR in HEK293T cells. **P < .01, ***P < .001. (E) P value was calculated by Student t test, and data are presented as the means ± SD from at least 3 independent experiments. CC, coiled-coil domain; Ctr, control; DAPI, 4',6-diamidino-2-phenylindole; PH, pleckstrin homology-like domain; Vec, vector; WCL, whole cell lysate.
recommended for metastatic CRC patients who experience progression on therapies such as 5-fluorouracil, leucovorin, oxaliplatin or capecitabine plus oxaliplatin without EGFR inhibitors, the results of our study justify a negative role of adjuvant therapy with respect to cetuximab efficacy in latent metastasis. As the direct target of cetuximab, EGFR expression has been proposed as a potential marker of clinical efficacy in early trials with cetuximab.\(^\text{39}\) However, the correlation between EGFR expression and cetuximab response is not evident in CRC,\(^\text{14,40,41}\) and the underlying mechanism that mediates resistance to EGFR-targeted therapy remains largely elusive.

Although EGFR is retained primarily along the plasma membrane, several studies have documented the nuclear localization of EGFR, wherein it functions as a transcription factor and a potential mechanism of resistance to EGFR inhibitor.\(^\text{42,43}\) Nuclear translocation of EGFR has been observed in cetuximab-resistant non–small cell lung cancer cells,\(^\text{42}\) but its role in CRC remains unclear, thus understanding the mechanism of EGFR nuclear translocation may enable the development of a new therapeutic strategy for CRC. Here, we found that PHLDB2 interacts with and stabilizes EGFR, facilitating its nuclear translocation and leading to cetuximab resistance in CRC cells. It has been determined that the phosphorylation-deficient variant S991A may impair receptor endocytosis, and phosphorylation of EGFR at site Ser991 determines the trafficking of EGFR.\(^\text{34}\) Consistently, we found that PHLDB2 reduced the
Figure 8. R1163A mutation diminishes PHLDB2-mediated cetuximab resistance both in vitro and in vivo. (A and B) Immunoblotting showing the phosphorylation and expression of the indicated proteins in WT or R1163A-mutated (RA) cells with or without 20 μg/mL cetuximab (CTX) treatment for 24 hours. (C) Cell nucleus/cytoplasm fractions were immunoblotted to determine EGFR localization in WT or R1163A-mutated cells. (D and E) MTT and colony formation assays were performed to determine the viability of the indicated cells in response to cetuximab treatment. (F and G) Soft agar colony formation assays were performed to determine the viability of the indicated cells in response to CTX (20 μg/mL) treatment. (H) A subcutaneous xenograft model was established in BALB/c nude mice injected with vector, PHLDB2 WT, and PHLDB2 RA NCI-H508 cells. One week after injection, the diameters of the tumors were measured every 3 days. Twenty-one days later, the mice were killed, and the tumors were dissected and photographed. (I and J) The protein levels of Ki67 and cleaved caspase 3 were detected by immunohistochemistry. Scale bars: 50 μm. **P < .01. (D–J) P values were calculated by Student t test. Data are presented as the means ± SD from at least 3 independent experiments. AKT, protein kinase B; C, cytoplasm; Ctr, control; ERK, extracellular signal–regulated kinase; N, nucleus; p-AKT, phosphorylated protein kinase B; p-ERK, phosphorylated extracellular signal–regulated kinase; Vec, vector.
binding affinity between EGFR and Rab11A, a well-known EGFR recycling protein involved in EGFR trafficking.20 Together, our findings provide a molecular basis for PHLDB2-mediated nuclear translocation of EGFR.

In summary, our data suggest a key role of PHLDB2 in mediating latent progression of CRC by modulating EGFR signaling. The up-regulation of PHLDB2 is attributed to METTL14-mediated m6A modification induced by chemotherapeutic agents. Mechanistically, PHLDB2 interacts with and stabilizes EGFR, facilitating its nuclear translocation and leading to cetuximab resistance. Thus, our findings implicate PHLDB2 as a potential marker and therapeutic target for latent metastatic CRC, providing a rationale for designing inhibitors targeting PHLDB2 as a novel approach for CRC treatment.

Materials and Methods

Antibodies and Reagents

The antibodies and reagents used in this study are listed in Table 1.

Table 1. Antibodies and Reagents

| Antibodies          | Source                  | Identifier      |
|---------------------|-------------------------|-----------------|
| PHLDB2 (1:200 [IF], 1:1000 [WB]) | Novus                  | NBP2-38238     |
| METTL3 (1:1000)     | Abcam                   | ab195352       |
| METTL14 (1:1000)    | Abcam                   | ab220030       |
| β-actin (1:2000)    | Santa Cruz Biotechnology | sc-69879      |
| ERK (1:1000)        | Cell Signaling Technology | 4695          |
| p-ERK (1:1000)      | Cell Signaling Technology | 4370          |
| JNK (1:1000)        | Cell Signaling Technology | 9252          |
| AKT (1:1000)        | Cell Signaling Technology | 9272          |
| p-AKT (1:1000)      | Cell Signaling Technology | 4060          |
| P38 (1:1000)        | Cell Signaling Technology | 8690          |
| p-P38 (1:1000)      | Cell Signaling Technology | 4511          |
| EGFR (1:1000)       | Cell Signaling Technology | 4267          |
| METTL3 (1:1000)     | Abcam                   | ab195352       |
| METTL14 (1:1000)    | Abcam                   | ab220030       |
| b-actin (1:2000)    | Santa Cruz Biotechnology | sc-69879      |
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| P38 (1:1000)        | Cell Signaling Technology | 8690          |
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| b-actin (1:2000)    | Santa Cruz Biotechnology | sc-69879      |
| ERK (1:1000)        | Cell Signaling Technology | 4695          |
| p-ERK (1:1000)      | Cell Signaling Technology | 4370          |
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| p-P38 (1:1000)      | Cell Signaling Technology | 4511          |
| EGFR (1:1000)       | Cell Signaling Technology | 4267          |
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| METTL14 (1:1000)    | Abcam                   | ab220030       |
| b-actin (1:2000)    | Santa Cruz Biotechnology | sc-69879      |
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| p-ERK (1:1000)      | Cell Signaling Technology | 4370          |
| JNK (1:1000)        | Cell Signaling Technology | 9252          |
| AKT (1:1000)        | Cell Signaling Technology | 9272          |
| p-AKT (1:1000)      | Cell Signaling Technology | 4060          |
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| METTL14 (1:1000)    | Abcam                   | ab220030       |
| b-actin (1:2000)    | Santa Cruz Biotechnology | sc-69879      |
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| p-P38 (1:1000)      | Cell Signaling Technology | 4511          |
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| METTL3 (1:1000)     | Abcam                   | ab195352       |
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| b-actin (1:2000)    | Santa Cruz Biotechnology | sc-69879      |
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| p-ERK (1:1000)      | Cell Signaling Technology | 4370          |
| JNK (1:1000)        | Cell Signaling Technology | 9252          |
| AKT (1:1000)        | Cell Signaling Technology | 9272          |
| p-AKT (1:1000)      | Cell Signaling Technology | 4060          |
| P38 (1:1000)        | Cell Signaling Technology | 8690          |
| p-P38 (1:1000)      | Cell Signaling Technology | 4511          |
| EGFR (1:1000)       | Cell Signaling Technology | 4267          |

Patients

Human CRC tissue samples were obtained from the BioBank of West China Hospital (Chengdu, China). To investigate the molecular characteristics of latent metastases, we selected 5 CRC cases with latent metastasis and collected their treatment-naive primary tumor and liver metastasis that occurred at least 6 months after primary surgery. Tumor tissues from 5 patients who underwent concurrent resection of the primary tumor and liver metastases without neoadjuvant therapy also were collected as a reference. To validate the expression of PHLDB2, 38 tumor samples from 31 metastatic CRC patients who underwent surgical resection also were obtained for immunohistochemistry (Figure 1F). In addition, tumor samples from 15 CRC patients administered cetuximab were included to address the association between PHLDB2 expression and cetuximab response (Figure 3K). Written informed consent was obtained from all patients. This study was approved by the Ethics Committee of West China Hospital, Sichuan University (2019 [38]; 2020 [374]).
Cell Lines

SW480, DLD-1, NCI-H508, and HEK293T cells were purchased from the American Type Culture Collection. Short tandem repeat analysis was performed for each of these cell lines. SW480, DLD-1, and HEK 293T cell lines were propagated in Dulbecco’s modified Eagle medium (Gibco, Shanghai, China); the NCI-H508 cell line was propagated in RPMI (Gibco, Shanghai, China). All cell lines were maintained in media supplemented with 10% fetal bovine serum (Biological Industries), 100 U/mL penicillin, and 100 μg/mL streptomycin (HyClone, Logan, UT) in a humidified incubator at 37°C under 5% CO₂.

Animal Studies

All animal procedures were approved by the Institutional Animal Care and Treatment Committee of Sichuan University. BALB/c nude female mice (age, 6 wk) were purchased from HFK Bioscience Co, Ltd (Beijing, China). Mice were housed in laminar flow cabinets under specific pathogen-free conditions with free access to water and food, under the room illumination cycle for a 12-hour light-dark cycle. For the subcutaneous CRC xenograft model, suspensions of the corresponding NCI-H508 cells were injected subcutaneously into the flanks (1 × 10⁷ tumor cells/100 μL phosphate-buffered saline [PBS] per spot; 6 mice in each group). When the tumor volume reached approximately 100 mm³, mice were administered 0.2 mL cetuximab (50 mg/kg in physiological saline, intraperitoneal injection, every 3 days). Animals were weighed, and the tumor size was measured using caliper measurements daily. Tumor volume was calculated using the following formula: volume = (L × W²)/2, where L = length and W = width. For spleen injection, mice were anesthetized with chloral hydrate and the spleen was exteriorized via a small left-side abdominal incision. DLD-1 cells (-vector or -PHLDB2) were injected slowly into the spleen (1 × 10⁶ tumor cells/50 μL PBS per spot). The muscle incision was closed with continuous absorbable sutures and the skin incision was closed with simple nonabsorbable sutures. To measure the luciferase intensity of injected cells, 2.25 μg/mL luciferin was injected intraperitoneally and luciferase activity was assessed 5 minutes after luciferin injection using an IVIS Spectrum Pre-clinical in vivo Imaging System (IVISSPE; PerkinElmer, Waltham, MA) machine.

Immunoblotting and Immunoprecipitation

Proteins were extracted using RIPA buffer (150 mmol/L NaCl, 50 mmol/L Tris-HCl [pH 7.5], 1 mmol/L EDTA, 0.25% Na-deoxycholate, and 1% NP-40) supplemented with protease inhibitors and phosphatase inhibitors (B14001 and B15001; Beyotime, Shanghai, China), and quantified using the BCA assay (23250; Thermo Fisher Scientific, Waltham, MA). Sodium dodecyl sulfate-polyacrylamide gel electrophoresis was performed to separate proteins, which then were transferred to polyvinylidene difluoride membranes (ISEQ00010; EMD Millipore, Burlington, MA). Next, the membranes were blocked in 5% fat-free milk for 2 hours and incubated with primary antibodies at 4°C overnight. After washing the membranes, secondary antibodies labeled with horseradish peroxidase (Santa Cruz Biotechnology, Dallas, TX) were applied at room temperature for 2 hours and the immunoreactivity was visualized by ECL (WBKLS0500; EMD Millipore, Burlington, MA). For immunoprecipitation, cells were collected and lysed with IP lysis buffer (100 mmol/L NaCl, 20 mmol/L Tris-HCl, 0.5 mmol/L EDTA, pH 7.4, and 0.5% NP-40). After centrifugation, the indicated antibodies (1 μg) were subjected to rotation overnight at 4°C. Then lysates were incubated with protein A Sepharose (17-0963-03; GE Healthcare, Chicago, IL) or protein G agarose (16-266; Millipore) for 3 hours, followed by centrifugation, washed 4 times using binding buffer (150 mmol/L NaCl, 20 mmol/L Tris-HCl, 0.5 mmol/L EDTA, pH 7.4, and 0.5% NP-40), and boiled with loading buffer for immunoblotting analysis.

MTT and Colony Formation Assay

For MTT assay, cells were seeded at 3 × 10³ cells/100 μL/well in 96-well plates and treated as indicated. A total of 10 μL MTT (5 mg/mL in PBS) was added to each well and cells were incubated at 37°C for 3 hours. To dissolve the precipitation, 150 μL dimethyl sulfoxide was added per well and the absorbance of the wells was measured at a 570-nm wavelength using a microplate reader. For the colony formation assay, cells were seeded at 5 × 10² cells/500 μL/well in 24-well plates for approximately 3 days and treated with the indicated agents. After incubation at 37°C for 10 days, colonies were fixed with 4% paraformaldehyde at room temperature for 30 minutes, stained with 0.2% crystal violet for 1 hour, and washed in double-distilled water (ddH₂O). For the soft agar colony formation assay, cells were suspended at low density in top agar (0.6%) with cell culture medium containing 10% fetal bovine serum, seeded on a layer of bottom agar (1.2%), and then grown for 3 weeks.

Measurement of Intracellular ROS Levels

The level of ROS in cells was measured by a ROS Assay Kit (S0033; Beyotime), following the manufacturer’s instructions. Briefly, cells were incubated with 10 μmol/L DCFH-DA (Sigma-Aldrich, Shanghai, China) in serum-free medium at 37°C for 15 minutes and then rinsed twice with PBS, followed by trypsinization and centrifugation. Then, cells were suspended in 500 μL PBS after 3 washes with PBS, and were analyzed with a BD FACS LSRII flow cytometer (BD Biosciences, San Jose, CA). Data analysis was performed with FlowJo VX software (Ashland, OR).

Cell Migration and Invasion Assays

Cell migration and invasion were performed by using 8.0-μm pore polycarbonate membrane inserts (CLS3422; Corning, Shanghai, China). A total of 1 × 10⁵ cells in serum-free culture medium were added to the chamber, and culture medium containing 10% fetal bovine serum as a chemoattractant was added to the 24-well plate. The chamber then was incubated at 37°C for 24 hours to allow cells to
penetrate an uncoated membrane or a Matrigel-coated membrane (cat. 356234; BD Biosciences, Heidelberg, Germany). Cells remaining on the upper surface of the membrane were removed with a cotton swab, and those on the underside of the membrane were fixed with 4% paraformaldehyde and stained with 0.2% crystal violet. Migrated cells were visualized using a DM2500 fluorescence microscope (Leica, Vista, CA).

**Plasmid Construction and Generation of Stable Cell Lines**

For PHLD2-Flag-tagged constructs, PHLD2 complementary DNA (cDNA) was inserted into the pcDNA3.1 vector (Addgene) with 3× Flag at the C-terminus. For EGFR–GFP-tagged constructs, EGFR cDNA was inserted into a modified pcDNA3.1 vector containing GFP tags. For Ubiquitin (UB)-Hemagglutinin (HA)-tagged constructs, UB cDNA was inserted into modified pcDNA3.1 vector containing HA tags. Single-point and truncated mutants were generated by the Fast Mutagenesis System (FM111; TransGen, Beijing, China). For the generation of PHLD2-overexpressed or PHLD2-silenced CRC cells, HEK293T cells were co-transfected with pSPAX2, pMD2.G, and pCDH-CMV-MCS-EF1-Puro-PHLDB2, or shPHLD2, or control shRNA plKO.1 plasmid. At 48 hours after transfection, lentivirus-containing medium was collected, filtered (0.45-μm filter), and supplemented with 8 mg/mL Polybrene (Sigma-Aldrich). CRC cells subsequently were transduced and supplemented with 8 mg/mL puromycin (540411; Calbiochem, San Diego, CA) for 2 weeks. The primer sequences are listed below:

- PHLDB2 1: CCGGCCCTGTAGTGATGTAA-
- PHLDB2 2: CCGGGCAGACGGCAATAATCTCTTACTCGAGTAA
- PHLDB2 3: CCTGGCCCTACGGCCGACAGA-3
- shPHLDB2 1: CCGGCCCTGTAGTGATGTAA-
- shPHLDB2 2: CCGGGCAGACGGCAATAATCTCTTACTCGAGTAA
- shPHLDB2 3: CCTGGCCCTACGGCCGACAGA-3

**RNA Sequencing Alignment and Quality Control**

RNA sequencing was performed with an Illumina HiSeq 2000 (Beijing Genomics Institute, Shenzhen, Guangdong, China). A total of 20 RNA sequencing libraries were aligned with Spliced Transcripts Alignment to a Reference (STAR) software (version 2.7.4a) in 2-pass mode. First alignment pass was used to identify nonannotated junctions in the input data, allowing for the construction of a genome index containing nonannotated junctions. The second pass alignment then was performed against the junction-aware index, allowing for a more sensitive recovery of nonannotated splice junction from the data. The human genome reference used was GRCh37.p13 and GENCODE v34 (GENCODE project, https://www.gencodegenes.org/) was used as the transcriptome reference.

**Quantification of Gene-Level and Transcript-Level Expression Gene Expression**

Quantification was performed using RSEM (version 1.3.3, https://deweylab.github.io/RSEM/) on STAR-aligned reads. The human reference sequence and gene transfer format (GTF) file used for build RSEM index was the same as described previously, and gene expression was quantified as transcripts per million.

**Differential Expression Analysis**

Differential expression analysis was performed using the DESeq2 R package (https://bioconductor.org/) to compare paired primary tumor, synchronous liver metastasis, and latent liver metastasis with available gene expression data. Briefly, each patient group was categorized as a synchronous and latent group according to the timing of metastasis. Sample type was defined as primary tumor and liver metastasis, respectively. The design formula was set as type + group + type:group. Differential expressed genes were extracted using the results function (ie, type:group interaction; SL vs P, SL/P; LL vs P, LL/P; and LL vs SL, LL/SL).

**Pathway Enrichment Analysis**

The genes significantly expressed between SL/P, LL/P, and LL/SL (adjusted P < .005) were analyzed for pathway enrichment using gprofiler (https://biit.cs.ut.ee/gprofiler/gost). All statistically significant pathways from the data source Kyoto Encyclopedia of Genes and Genomes (KEGG) were obtained using a threshold of 0.05 of the g:SCS (Set Counts and Sizes) threshold.

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The genes significantly expressed between SL/P, LL/P, and LL/SL (adjusted P < .005) were analyzed for pathway enrichment using gprofiler (https://biit.cs.ut.ee/gprofiler/gost). All statistically significant pathways from the data source Kyoto Encyclopedia of Genes and Genomes (KEGG) were obtained using a threshold of 0.05 of the g:SCS (Set Counts and Sizes) threshold.
**Immunofluorescence**

Cells were seeded onto the glass coverslips (WHB-24-CS; WHB Scientific, Shanghai, China) in 24-well plates. Cells were fixed with 4% paraformaldehyde in PBS for 30 minutes, permeabilized, and blocked with 0.3% Triton X-100 (T8200; Solarbio, Beijing, China) and 5% goat serum (G9023; Sigma-Aldrich) for 1 hour. After the incubation with primary antibodies at 4°C overnight, cells were incubated further with the appropriate secondary antibodies (35552; Invitrogen for Dylight 594-conjugated goat anti-rabbit IgG, and 35511; Invitrogen for Dylight 488-conjugated goat antimouse IgG, Carlsbad, CA) for 2 hours at room temperature. Nuclei were finally stained with 4',6-diamidino-2-phenylindole (C0060; Solarbio) for 5 minutes at a 1:5000 dilution. Fluorescence images were captured using confocal laser scanning microscopy (Carl Zeiss Microimaging, Thornwood, NY) fitted with a 63× oil immersion objective. Image analysis was performed with ImageJ software (National Institutes of Health, Bethesda, MD).

**Immunohistochemistry**

For immunohistochemistry (IHC), paraffin-embedded slides were deparaffinized in xylene, followed by hydration in ethanol with gradient-decreased concentrations. Endogenous peroxidase was blocked by 3% H2O2. Then, the sections were boiled in antigen retrieval buffer for 3 minutes within a pressure cooker. Next, slides were incubated with the primary antibody: PHLDB2 (1:100, NBP2-38238; Novus, Littleton, CO), Ki67 (1:100, ab16667; Abcam, Cambridge, UK), or cleaved caspase-3 (1:100, 9664S; Cell Signaling Technology, Shanghai, China) at 4°C overnight, followed by treatment with MaxVision horseradish-peroxidase solution (cat. 5020; MXB Biotechnology) for 30 minutes at room temperature. After 3 washes with PBS, sections were stained with 3,3’-diaminobenzidine tetra hydrochloride peroxidase substrate (0031; MXB Biotechnology, Fuzhou, China) and counterstained with hematoxylin. Images were captured by a Pannoramic MIDI Slide scanner (3D HISTECH, Budapest, Hungary). Ki67 and cleaved caspase-3 staining was quantified by calculating positively stained cells in at least 5 randomly chosen high-power fields of each sample.

**Immunoprecipitation Followed by MS**

Cell lysates were prepared using IP lysis buffer (100 mmol/L NaCl, 20 mmol/L Tris-HCl, 0.5 mmol/L EDTA, pH 7.4, and 0.5% NP-40). After centrifugation to remove insoluble materials, lysates were incubated with PHLDB2 antibody at 4°C overnight. The next day, lysates were incubated with protein G-Agarose beads (16-266; Millipore) for 3 hours. The immunoprecipitated proteins were subjected to short-gel sodium dodecyl sulfate-polyacrylamide gel electrophoresis and stained for 20 minutes with Coomassie brilliant blue G-250 (175 A100615; Sangon Biotech, Shanghai, China). The protein bands of interest were excised, carefully washed, and dehydrated by acetonitrile (A955-4; Fisher Scientific). Then, 10 mmol/L dithiothreitol (D0632, Sigma-Aldrich) was applied for reduction and 55 mmol/L iodoacetamide (V900335; Sigma-Aldrich) for alkylation. Proteins were digested with trypsin (V5071; Promega, Madison, WI) for 12 hours.

After being desalted using C18 ZipTip (Millipore) according to the manufacturer’s instructions, the peptide samples were loaded onto a 75 μm × 2 cm trap column and a 75 μm × 12 cm analytical column, which was packed with C18 resin (200 A, 5 μm; Michrom Bioresources, Auburn, CA) in-house. LC-MS/MS analysis was executed using an EASY-NLC 1000 nanoflow LC instrument coupled to a Q Exactive quadrupole-Orbitrap mass spectrometer (Thermo Fisher Scientific). Peptide samples were analyzed with a 65-minute gradient from 4% to 90% high-performance liquid chromatography buffer B (95% acetonitrile, 0.1% formic acid) at a flow rate of 300 nL/min. Data-dependent acquisition was performed in positive ion mode. Survey full-scan MS spectra (from m/z 350-1800) were acquired in the Orbitrap with a resolving power of 70,000 at m/z = 200. The automatic gain control value setting was set at 3e6, with maximum fill times of 20 ms. For MS/MS scans, the top 20 most intense parent ions were selected with a 1.6 m/z isolation window and fragmented with a normalized collision energy of 27%. The automatic gain control value for MS/MS was set to a target value of 1e5, with a resolution of 17,500 and a maximum fill time of 64 ms. Parent ions with a charge state of z = 1, 8, or with unassigned charge states were excluded for fragmentation. A dynamic exclusion period for the data-dependent scan was 50 seconds. All the raw files were searched against the Swiss-Prot human protein sequence database (updated in April 2019, 20,431 sequences) in Maxquant (version 1.6, https://maxquant.org). The precursor peptide mass tolerance was 10 ppm and a fragment ion mass tolerance was 0.02 daltons. Two missed trypsin cleavages were allowed. Cysteine carbamidomethylation was set as a fixed modification. Oxidation of methionine and protein N-terminal acetylation were set as variable modifications. A label-free quantification algorithm was used for protein quantification. Peptides with less than 1% false-discovery rate were chosen. A minimum of 7 unique peptides at the protein level within the full data set was required for further data processing.

**Duolink In Situ Proximity Ligation Assay**

Cells were seeded onto glass coverslips (WHB-24-CS; WHB Scientific) in 24-well plates, and then fixed with 4% paraformaldehyde in PBS for 30 minutes, permeabilized, and blocked with 0.3% Triton X-100 (T8200; Solarbio) and 5% goat serum (G9023; Sigma-Aldrich) for 1 hour. Cells then were incubated with mouse EGFR antibody (1:100; Santa Cruz Biotechnology) and rabbit PHLDB2 antibody (1:100, NBP2-38238; Novus) or Flag antibody (1:100, 14793S; Cell Signaling Technology) at 4°C overnight. The fluorescence signals were detected by the Duolink in situ proximity ligation assay probe (DUO92101-1KT; Sigma-Aldrich, Shanghai, China) according to the manufacturer’s instructions and visualized using confocal laser scanning
microscopy (Carl Zeiss Microimaging) fitted with a 63× oil immersion objective. Image analysis was performed with ImageJ software.

**Public Data Analysis**

For Gene Expression Omnibus (GEO) data, the gene expression data were obtained from GSE17536 and GSE28722 as well as their corresponding clinical data using the GEO query R package. For TCGA data, the mRNA expression of each tumor was obtained from the cbioportal feature mRNA Expression, RSEM (Batch normalized from Illumina HiSeq_RNASeqV2), while survival data were obtained from the TCGA Pan-Cancer Clinical Data Resource. The mRNA expression (transcripts per million) of CRC cell lines was obtained from the Broad Institute Cancer Cell Line Encyclopedia, and cetuximab sensitivity was obtained based on published reports.23

**Statistical Analysis**

All data were analyzed by GraphPad Prism 8.0 software (San Diego, CA). Survival analysis was performed using the log-rank test. The survcurve function of the survminer package in R (https://cran.r-project.org/) was used to determine the optimal cut-off point based on the maximally selected log-rank statistics. The minimal proportion of observations per group was set to 30% to avoid too few patients in a certain group. Data are representative of at least 3 biologically independent experiments. Comparisons between 2 groups were performed by a 2-tailed Student t test. For 3 or more group analyses, the 1-way analysis of variance multiple comparison test was used. Pearson correlation and linear regression were used to determine the concordance. Data are expressed as means ± SD.

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Received September 7, 2021. Accepted December 10, 2021.

Correspondence
Address correspondence to: Hai-Ning Chen, MD, PhD, Department of Gastrointestinal Surgery, State Key Laboratory of Biotherapy and Cancer Center, West China Hospital, Sichuan University, Chengdu, 610041, P.R. China. Tel: +86-18980606468, e-mail: hncchen@scu.edu.cn; or Canhua Huang, PhD, State Key Laboratory of Biotherapy, West China Hospital, and West China School of Basic Medical Sciences & Forensic Medicine, Sichuan University, No. 17, Section 3, South Renmin Rd, Chengdu, 610041, P.R. China. Tel: +86-13258370346, e-mail: hcanhua@hotmail.com; fax: +86-28-85164060.

Acknowledgments
The authors would like to thank Ping Fan of West China Biobanks, Department of Clinical Research, West China Hospital of Sichuan University for biospecimen collection, processing, quality control, and storage.

Ethics Approval
This study was approved by the Ethics Committee of West China Hospital, Sichuan University (2019 [338]; 2020 [374]). The study was performed in accordance with the Declaration of Helsinki.

Data Availability Statement
Further information and requests for resources and reagents should be directed to and will be fulfilled by the Lead Contact, Hai-Ning Chen (hncchen@scu.edu.cn). Most data supporting the findings of this study are available within the article and online supplementary files. The raw sequencing data from this study have been deposited in the Genome Sequence Archive in BIG Data Center, Beijing Institute of Genomics, Chinese Academy of Sciences, under accession number PRJCA007055 (BioProject), which can be accessed at http://bigd.big.ac.cn/gsa-human. All other data are available from the corresponding author upon reasonable request.

CRediT Authorship Contributions
Hai-Ning Chen, PhD, MD (Conceptualization: Lead; Supervision: Equal; Writing – review & editing: Equal)
Maochao Luo (Conceptualization: Supporting; Investigation: Equal; Writing – original draft: Lead)
Zhao Huang (Conceptualization: Supporting; Investigation: Equal)
Xingyue Yang (Investigation: Equal; Writing – original draft: Equal)
Yan Chen (Investigation: Equal)
Jingwen Jiang (Investigation: Supporting)
Lu Zhang (Investigation: Supporting)
Li Zhou (Investigation: Supporting)
Siuyuan Qin (Investigation: Supporting)
Ping Jin (Investigation: Supporting)
Shuyue Fu (Investigation: Supporting)
Liyuan Peng (Investigation: Supporting)
Bowen Li (Investigation: Supporting)
Yongting Fang (Investigation: Supporting)
Wenchen Pu (Methodology: Supporting)
Yanqiu Gong (Methodology: Supporting)
Yu Liu (Methodology: Supporting)
Zhixiang Ren (Formal analysis: Supporting)
Oui-Luo Liu (Investigation: Supporting)
Cun Wang (Resources: Supporting)
Fangqiong Xiao (Resources: Supporting)
Du He (Resources: Supporting)
Hongying Zhang (Resources: Supporting)
Changlong Li (Resources: Supporting)
Heng Xu (Funding acquisition: Supporting; Resources: Supporting)
Lunzhi Dai (Methodology: Supporting)
Yong Peng (Writing – review & editing: Supporting)
Zong-Gung Zhou (Funding acquisition: Supporting; Resources: Equal)
Canhua Huang (Conceptualization: Equal; Funding acquisition: Lead; Supervision: Equal; Writing – review & editing: Supporting)

Conflicts of interest
The authors disclose no conflicts.

Funding
This work was supported by grants from the National Key Research and Development Project of China (2020YFA05090400 and 2020YFC2002705), the national natural science foundation of china (81702378, 82073246, 81821002, 81790251, 82130082, and 82003098), Guangdong Basic and Applied Basic Research Foundation (2019B030302012), Sichuan Science and Technology Program (22CXRJ0248, 2020YJ0105, and 2020YJ0094), and the 1,3,5 project for disciplines of excellence, West China Hospital, Sichuan University (201610S, ZYG20006, and ZYYC20003).