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Original Articles

The PEAK1–PPP1R12B axis inhibits tumor growth and metastasis by regulating Grb2/PI3K/Akt signalling in colorectal cancer

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ABSTRACT

Pseudopodium enriched atypical kinase 1 (PEAK1), a novel non-receptor tyrosine kinase, was recently implicated in cancer pathogenesis. However, its functional role in colorectal cancer (CRC) is not well known. Herein, we demonstrated that PEAK1 was frequently downregulated in CRC and significantly associated with tumor size, differentiation status, metastasis, and clinical stage. PEAK1 overexpression suppressed CRC cell growth, invasion, and metastasis *in vitro* and *in vivo*, whereas knockout had the opposite effects. Further evaluation revealed that PEAK1 expression was positively correlated with protein phosphatase 1 regulatory subunit 12B (PPP1R12B) in CRC cell lines and clinical tissues, and this protein was found to suppress activation of the Grb2/PI3K/Akt pathway. Moreover, PPP1R12B knockdown markedly abrogated PEAK1-mediated tumor suppressive effects, whereas its upregulation recapitulated the effects of PEAK1 knockout on cell behaviours and the activation of signalling. Mechanistically, PI3K and Akt inhibitors reversed impaired the effect of PEAK1 function on cell proliferation, migration, and invasion. Our results provide compelling evidence that the PEAK1–PPP1R12B axis inhibits colorectal tumorigenesis and metastasis through deactivation of the Grb2/PI3K/Akt pathway, which might provide a novel therapeutic strategy for CRC treatment.

1. Introduction

Colorectal cancer (CRC) is one of the most prevalent carcinomas worldwide [1]. Distant metastasis, one of the six initial cancer hallmarks [2], is responsible for as much as 90% of cancer-related mortality [3,4]. Although primary tumors are often treated by radical surgery or other therapies, advanced CRC is associated with limited options regarding targeted agents and conventional chemo-/radiotherapy. CRC progression and metastasis is influenced by a series of well-characterized processes, such as impaired tumor suppressor genes function, epithelial-to-mesenchymal transition (EMT) and the activation of protein tyrosine kinases (PTKs)-mediated signalling [5]. The understanding of the mechanisms underlying these processes has provided the basis for the development of therapies to target metastasis. However, metastasis requires multiple steps and remains one of the most complicated aspects of cancer progression; the exact molecular signals and key factors underlying each step have not been completely explored.

PEAK1 (pseudopodium enriched atypical kinase 1, also known Sgk269) is a member of the new kinase family three of protein kinases that mediates interactions with various signalling pathways, such as p130Cas/Crk/Rac1 and Ras/Raf/ERK [6,7]. It is also associated with cytoskeletal organization, cell migration, and focal adhesion turnover [8,9]. Recent studies have emerged supporting a functional role for PEAK1 in the development of several human malignancies. Increased PEAK1 expression has been detected in pancreatic cancer [10], and was shown to be critical for tumor growth, metastasis, and therapy resistance [10–12]. Additionally, its overexpression was also found in basal breast cancer cell lines and a subset of primary breast cancers

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Abbreviations

CCK-8	Cell Counting Kit-8
CRC	colorectal cancer
EGFR	epidermal growth factor receptor
EMT	epithelial-to-mesenchymal transition
ERK	extracellular signal-regulated kinase
IHC	immunohistochemistry
NRTK	non-receptor PTKs
PBS	phosphate-buffered saline
PEAK1	pseudopodium enriched atypical kinase 1, also known Sgk269
PPP1R12B	protein phosphatase 1 regulatory subunit 12B
PTK	protein tyrosine kinase
TGFβ	transforming growth factor β

[13]; it is also thought to act as a molecular switch for transforming growth factor β (TGFβ)-induced EMT [14,15]. These studies suggest that PEAK1 might play an oncogenic role in pancreatic and breast cancers. However, PEAK1 was markedly decreased in gastric cancer specimens compared to expression in non-neoplastic gastric epithelium tissues, and its expression was positively correlated with E-cadherin and favourable patient prognosis, indicating that PEAK1 might suppress gastric cancer metastasis by negatively regulating EMT [16].

Given the controversial role of PEAK1 in different cancers, our aim was to delineate the functional role of this protein in CRC using clinical patient samples, *in vitro* cell culture system and *in vivo* mouse model. Our findings would provide new insights into the functional role of PEAK1 in tumorigenesis and metastasis and suggest a promising therapeutic strategy for CRC treatment.

2. Materials and methods

2.1. Cell culture and tissue specimens

SW480, SW620, HT29, HCT116, T84, LoVo and Caco-2 were obtained from the Cell bank of Chinese Academy of Sciences (Shanghai, China), and maintained in Leibovitz's L-15, McCoy's 5A, F-12K or MEM medium (GIBCO Laboratories, USA) supplemented with 10% foetal bovine serum (FBS, Invitrogen, USA) at 37 °C with 5% CO_2.

In this study, 124 formalin-fixed paraffin-embedded tissues (including 84 tumor and 40 normal tissues), and 27 pairs of primary CRC tissues and matched normal tissue samples were obtained from the Affiliated Hospital of Zunyi Medical University, and the Affiliated Zhongda Hospital of Southeast University between 2015 and 2017. No cases received radiotherapy or chemotherapy before surgery. All patients provided written informed consent before surgery, and the use of tissues was approved by the Ethics Committee of the Affiliated Zhongda Hospital of Southeast University, and the Affiliated Hospital of Zunyi Medical University.

2.2. Plasmids, transfection, and lentivirus infection

N-terminal HA-tagged full-length human PEAK1 cDNA (WT) and *PEAK1* △Shc and △Crk mutants (Genscript Biotechnology, China) were subcloned into pLVX-IRES-Puro lentiviral vectors (Clontech, USA). The lenti-CAS9-sgRNA system for PEAK1 knockout and control lentivirus vectors were constructed by GeneChem (Shanghai, China). For lentiviral infection, different CRC cells were infected with lentiviral vectors at an MOI of approximately 60 with 6 μg/ml polybrene for 16 h. Then, the culture medium was removed and fresh medium was added; 48 h post-infection, stable pools of cells were selected with 5 μg/ml puromycin (Invitrogen, USA). Stable overexpressing and knockout cell lines were generated as previously described [17]. To evaluate PPP1R12B expression, *PPP1R12B* cDNA was synthesized and subcloned into pcDNA3.1 (Genscript Biotechnology, China). Different CRC cell lines with ectopic expression of PPP1R12B were achieved by pcDNA3.1-PPP1R12B transfection with Lipofectamine® 3000 (Invitrogen, USA) according to the manufacturer's instructions. The empty pcDNA vector was used as a negative control. In addition, PPP1R12B-specific siRNA oligonucleotides (Cat. no. AM16708, Assay ID 144389, Invitrogen, USA) and negative control siRNA (Cat. no. AM4611, Invitrogen, USA) were transfected into CRC cells using Lipofectamine RNAiMAX (Invitrogen, USA) in OptiMEM (Invitrogen, USA) following the manufacturer's protocol.

2.3. Quantitative real-time PCR (qRT-PCR)

Total RNA was extracted using Trizol (Invitrogen, USA) and reverse transcribed into cDNA using a RT reagent Kit (TakaRa, Japan) according to the manufacturer's instructions. qRT-PCR was performed as previously described [18]. Briefly, PrimeScript RT Master Mix (TaKaRa, Japan) and SYBR Premix Ex Taq II (TaKaRa, Japan) were used to detect mRNA expression. The mix was preheated at 95 °C (45 s), amplified at 95 °C (10 s) and 60 °C (40 s) for 40 cycles. The $2^{-\Delta\Delta Ct}$ method was used to calculate expression relative to the endogenous control. The primer sequences are listed in Supplemental Table 1.

2.4. Tissue immunohistochemistry

Immunohistochemistry (IHC) was performed on formalin-fixed paraffin-embedded sections; immunostaining intensity and average percentage of positive cells were evaluated as previously reported [18,19]. The quantification of vessel density was determined as described [20]. The primary antibodies are described in Supplemental Table 2.

2.5. Western blot analysis

Western blot analysis was performed as previously described [18]. The primary antibodies are described in Supplemental Table 2.

2.6. Microarray analysis

PEAK1-overexpressing LoVo cells and negative control cells were used for gene expression profiling. Total RNA was isolated from three independent cultures using Trizol (Invitrogen, USA) and the miRNeasy mini kit (Qiagen, Germany) according to manufacturer's instructions, amplified and transcribed into fluorescent cRNA using the Quick Amp Labeling Kit (Agilent Technologies, USA). RNA quality and quantity were measured using the Nanodrop ND-1000 (Nanodrop Technologies, USA) and RNA integrity was determined by gel electrophoresis. Microarray analysis of mRNA profiles using Agilent Array platforms and the identification of differentially expressed genes were performed by KangChen Bio-tech (Shanghai, China) as previously reported [21].

2.7. Cell proliferation and clonogenic assays

Cell proliferation was assessed by performing Cell Counting Kit-8 (CCK-8) assays (Dojindo, Kumamoto, Japan), as previously described [22]. Briefly, infected or transfected CRC cells were seeded in 96-well plates (1×10^4 per well) in triplicate. After incubation at 37 °C for 1–5 or 1–4 days, 10 μl of CCK-8 solution was added to each well, and cells were incubated for another 3 h at 37 °C; the plates were gently shaken, and the absorbance values were measured at 450 nm using an ELISA reader (BioTek, Winooski, VT, USA) according to the manufacturer's

instructions. The clonogenic assay was performed as described previously [23].

2.8. Cell migration, invasion and wound-healing assays

Cell migration and invasion assays were conducted using 24-well plates and 8-μm-pore size polycarbonate membranes (Corning Costar, Lowell, MA, USA). For this, 5×10^4 cells were seeded onto the upper chamber with or without Matrigel (BD Biosciences, SanJose, CA, USA), as previously reported [24]. Three randomly selected fields-of-view from five areas, specifically upper, lower, left, right, and middle, were analysed to quantify the average numbers of invading/migrating cells. DMSO (vehicle), LY294002, or ipatasertib was added to both the top and bottom of the transwell. For wound-healing assays, CRC cells were cultured in six-well plates until they are confluent and scratched with a 10-μl pipette tip. The previous medium was replaced with fresh medium containing 1% FBS. Cells were photographed at 0, 6, 12 and 24 h after scratching.

2.9. Immunofluorescence analysis

PEAK1-overexpressing LoVo and HCT116 cells and corresponding control cells were fixed with 4% paraformaldehyde at room temperature for 15 min. After washing three times in phosphate-buffered saline (PBS), CRC cells were stained as previously described [25]. All pictures were collected using a confocal microscope (Olympus, Tokyo, Japan).

2.10. Sphere-forming assays

Cells ($2–5 \times 10^3$/well) were seeded on six-well ultra-low attachment plates as described previously [26]. Tumor spheres > 75 μm in diameter were counted after 15 days using an optical microscope.

2.11. Animal experiments

Five week-old female BALB/c nude mice were obtained from Yangzhou University Animal Center (Yangzhou, China). For tumor growth analysis, stable PEAK1 overexpressing and control LoVo cells (2×10^6) were harvested during the log phase of growth and re-suspended in 100 μl of cold PBS, and then implanted subcutaneously into BALB/c nude mice (six per group). Tumor length (L) and width (W) were measured every 7 days using digital Vernier calipers. Tumor volumes were determined by external measurements according to the formula $W^2 \times L/2$ [27]. To induce experimental liver metastasis, 1×10^6 tumor cells (LoVo-PEAK1 and LoVo-Ctrl) suspended in 100 μl of PBS were injected into 6-week-old BALB/c nude mice (six per group) via the lateral tail vein. All mice were sacrificed under general anaesthesia 5 weeks after injection. Primary tumors and liver tissues were resected, fixed, and embedded in paraffin for histologic haematoxylin and immunohistochemistry analyses. All animal experiments were conducted according to protocols approved by the Animal Care and Ethical Committee of Medical School of Southeast University.

2.12. Statistical analysis

SPSS V19.0 software was used for statistical analysis. PEAK1 mRNA and protein expression in paired frozen tissues were evaluated with paired t-tests. Pearson's $\chi 2$ test was used to compare the correlation between PEAK1 expression and clinicopathologic features. Two-tailed Student's t-test and one-way analysis of variance analysis were used to determine significance, comparing two and multiple groups, respectively. Each experiment was repeated at least three times and results are presented as the mean ± SD. Differences with $P < 0.05$ were considered significant.

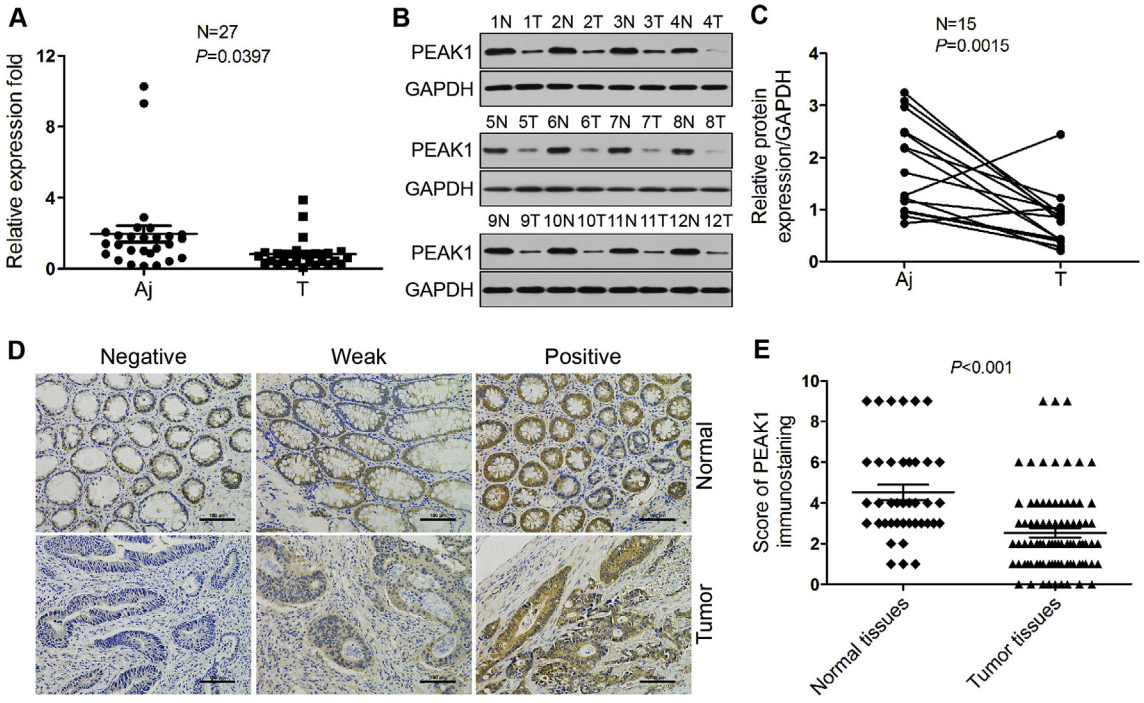

Fig. 1. PEAK1 is aberrantly downregulated in CRC tissues. (A) The expression of *PEAK1* mRNA in 27 pairs of CRC tissues and adjacent normal tissues was assessed by qRT-PCR. GAPDH was used as an internal control. Aj, adjacent normal tissue; T, CRC tissue. Error bars show mean ± SD. The *P* value is for paired *t*-test. (B) Representative data of PEAK1 protein expression in 15 paired CRC tissues and adjacent normal tissues were detected via western blot analysis. N, adjacent normal tissue; T, CRC tissue. (C) Relative PEAK1 protein expression levels in 15 paired CRC tissues and noncancerous tissues. Aj, adjacent normal tissue; T, CRC tissue. Error bars show mean ± SD. The *P* value is for paired *t*-test. (D) IHC analysis of PEAK1 expression in 40 adjacent normal tissues and 84 CRC tissues. Scale bar, 100 μm. (E) Results of immunohistochemistry staining were evaluated by the staining scores. Error bars show mean ± SD. The *P* value is for unpaired *t*-test.

3. Results

3.1. PEAK1 is aberrantly downregulated in human CRC tissues

To investigate the expression and clinical significance of PEAK1 in CRC, we first assessed *PEAK1* mRNA levels in 27 pairs of CRC specimens and adjacent normal tissues. Results showed that *PEAK1* expression was markedly decreased in tumor tissues compared to that in the corresponding non-cancerous tissues (Fig. 1A). Next, we detected PEAK1 protein levels in 15 paired tumor and adjacent normal tissues from 27 paired clinical specimens. As shown in Fig. 1B and C, results were similar to mRNA levels. Furthermore, IHC staining suggested that PEAK1 was localized to both the cytoplasm and membranes (Fig. 1D), and was clearly reduced in CRC tissues, as compared to expression in normal colorectal tissues (Fig. 1E, Supplemental Table 3). Clinicopathological characteristics of 84 CRC patients showed that low PEAK1 expression was obviously associated with tumor size and differentiation status, lymph node metastasis, distant metastasis and clinical stage (Table 1). Collectively, these results strongly indicate that PEAK1 downregulation might contribute to CRC progression.

3.2. PEAK1 suppresses cell proliferation and clonogenic ability in vitro

Subsequently, we tested seven human CRC cell lines (SW480, SW620, HT29, T84, Caco-2, HCT116 and LoVo) for PEAK1 expression. qRT-PCR and western blot assays showed that primary cancer cell lines SW480 and HT29 expressed higher level of PEAK1 than the highly metastatic cell lines LoVo and HCT116 (Fig. 2A and B). Therefore, we stably expressed PEAK1 in LoVo and HCT116 cells, whereas PEAK1 was stably knocked down in SW480 and HT29 cells using the CRISPR-Cas9 system. Altered PEAK1 expression was confirmed by western blot analysis (Fig. 2C).

Cell proliferation and clonogenic abilities were significantly decreased following ectopic upregulation of PEAK1 in LoVo and HCT116 cells (Fig. 2D and E), whereas PEAK1 knockout obviously increased cell proliferation and colony formation in SW480 and HT29 cells, as compared to those in corresponding control cells (Fig. 2F and G). These data suggest that PEAK1 expression impairs the proliferation and clonogenic abilities.

3.3. PEAK1 expression inhibits cell migration, invasion and EMT in vitro

Next, we assessed the effect of PEAK1 on CRC cell migration and invasion. Transwell assays showed that PEAK1 overexpression in LoVo and HCT116 cells could markedly suppress cell migration and invasion (Fig. 3A and B). Conversely, PEAK1 knockout significantly conferred migrative and invasive abilities to SW480 and HT29 cells (Fig. 3C and D). Scratch-wound assays were performed to further confirm the inhibitory effect of PEAK1 on CRC cell migration, and the results are shown in Supplemental Fig. 1.

EMT is crucial for cancer cell metastasis [28]. E-cadherin and β-catenin are epithelial markers and usually inhibited by EMT, whereas the expression of mesenchymal markers such as N-cadherin and vimentin is enhanced when epithelial cells undergo EMT [29,30]. In the study, we found that PEAK1 upregulation in LoVo and HCT116 cells resulted in low expression of N-cadherin and vimentin and high expression of E-cadherin and β-catenin (Fig. 3E, Supplemental Fig. 2A), whereas PEAK1 knockout in SW480 and HT29 cells led to the opposite effects (Fig. 3F, Supplemental Fig. 2B). Similar results for E-cadherin and vimentin expression were obtained by immunofluorescence (Fig. 3G). Together, these results reveal that PEAK1 has a tumor suppressive role in CRC cell metastasis by inhibiting EMT-like behaviours.

3.4. PEAK1 overexpression suppresses tumorigenesis and metastasis in a xenograft model

Based on cell-based results, we speculated that PEAK1 overexpression could suppress tumor growth and metastases in animals. To explore this, LoVo cells stably overexpressing PEAK1 or controls were subcutaneously injected into the flanks of nude mice. As predicted, PEAK1-overexpressing LoVo cells resulted in markedly reduced tumor volumes and weights and exhibited decreased proliferation rates, as compared to those in the control group (Fig. 4A–C). Moreover, the control group exhibited higher ki67 expression compared to that in the PEAK1 overexpression group (Fig. 4D, Supplemental Fig. 3A). Notably, tumors in the control group appeared more reddish than those formed by LoVo-PEAK1 cells (Fig. 4A). We further confirmed that vessel density was markedly decreased in PEAK-overexpressing tumors based on the detection of $CD31^{+}$ vascular structures, as compared to those in the LoVo-Ctrl group (Supplemental Figs. 3B and C).

Then, we used experimental metastasis animal models to determine if PEAK1 inhibits tumor metastasis *in vivo*. LoVo control cells or PEAK1-overexpressing cells were intravenously injected into nude mice via the tail vein. At the fifth week, mice were sacrificed, livers were collected, and haematoxylin and eosin (HE) staining was performed to evaluate tissue morphology (Fig. 4E). In controls, marked liver metastases and more tumor nodules were detected, whereas PEAK1 overexpression resulted in significant reduction in metastatic nodules (Fig. 4F). These *in vivo* results corroborated *in vitro* data and suggest a crucial tumor suppressive role for PEAK1.

3.5. Identification of PEAK1 as a positive regulator of PPP1R12B in human CRC

To investigate the mechanisms underlying the tumor suppressive effects of PEAK1, we performed global gene expression profiling. The data set can be accessed under the GEO accession number GSE108947.

Table 1
Correlation between PEAK1 expression and clinicopathological characteristics of CRC patients.

Characteristics	Cases	PEAK1 immunostaining		P value
		Negative	Positive	
Gender				0.349
Male	52 (61.9%)	33	19	
Female	32 (38.1%)	17	15	
Age				0.770
≤60	33 (39.3%)	19	14	
> 60	51 (60.7%)	31	20	
Tumor size				**0.010**
≤4 cm	45 (53.6%)	21	24	
> 4 cm	39 (46.4%)	29	10	
Histology				0.974
Tubular	74 (88.1%)	44	30	
Mucinous, papillary	10 (11.9%)	6	4	
Tumor location				0.753
Colon	28 (33.3%)	16	12	
Rectal	56 (66.7%)	34	22	
Tumor differentiation				**0.032**
Well-moderate	61 (72.6%)	32	29	
Poor	23 (27.4%)	18	5	
Lymph node metastasis				**0.007**
Absent	55 (65.5%)	27	28	
Present	29 (34.5%)	23	6	
Distant metastasis				**0.001**
Negative	67 (79.8%)	34	33	
Positive	17 (20.2%)	16	1	
TNM stage				**0.008**
I-II	58 (69.0%)	29	29	
III-IV	26 (31.0%)	21	5	

TNM, tumor, node, metastasis.

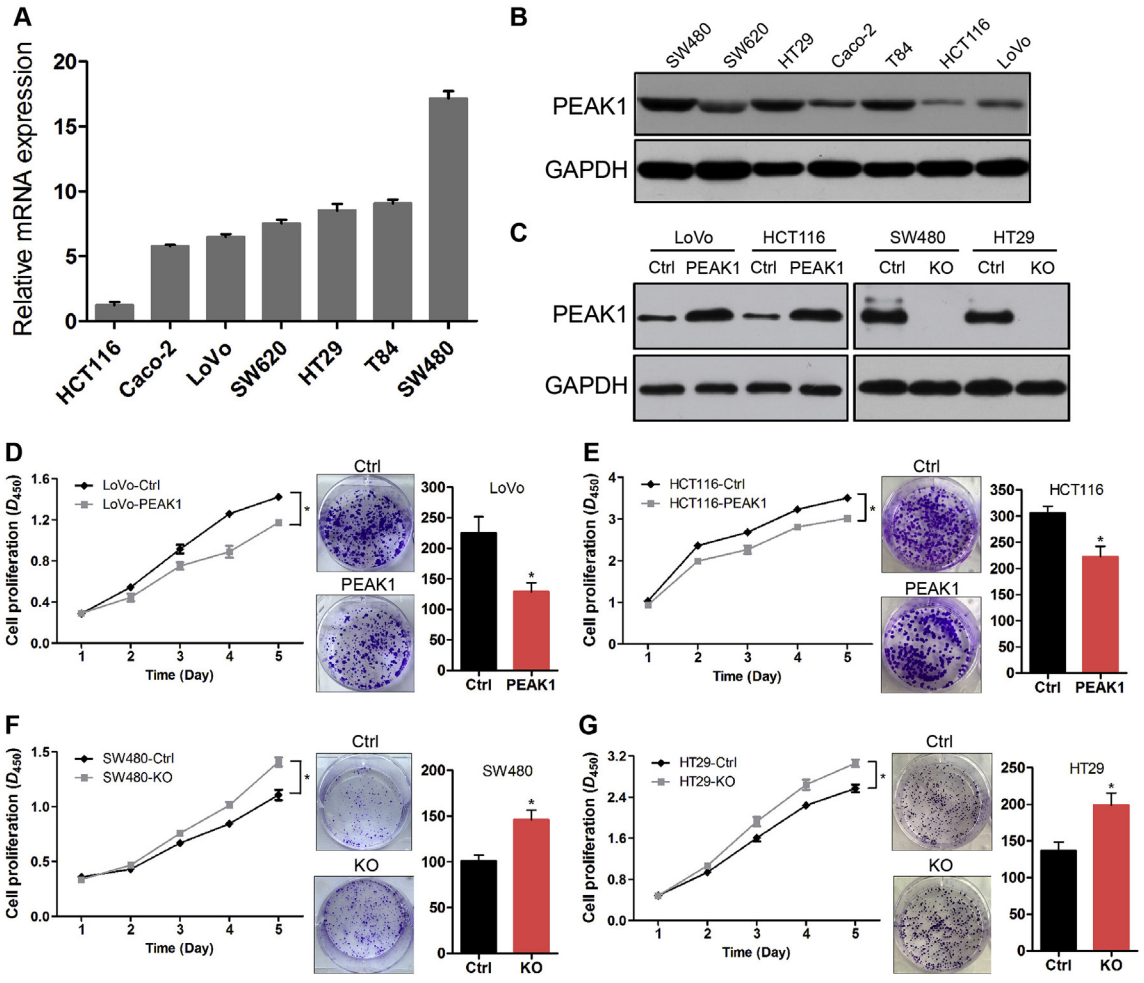

Fig. 2. PEAK1 is decreased in highly invasive cancer cell lines and inhibits cell proliferation and colonigenic abilities. (A) The relative expression of *PEAK1* mRNA in seven human CRC cell lines was detected by qRT-PCR. GAPDH was used as an internal control. (B) The expression of PEAK1 protein in seven CRC cell lines was measured via western blot analysis. (C) After cells were infected with PEAK1-overexpression and knockout lentiviral vectors as well as corresponding control vectors, the levels of PEAK1 protein expression were detected through western blot analysis. (D, E) Overexpression of PEAK1 suppressed LoVo and HCT116 cells growth *in vitro* by using CCK-8 and clonogenic assays. (F, G) PEAK1 knockout enhanced SW480 and HT29 cells proliferation and colonigenic abilities *in vitro* (two clones, D and E). These data represent at least three independent experiments. Error bars show mean ± SD. $^*P < 0.05$.

Altered gene expression profiles in PEAK1-overexpressing LoVo cells are presented as a heatmap (Fig. 5A). We found that PEAK1 could potentially upregulate 111 genes and downregulate 82 genes (Supplemental Fig. 4). Among these, 27 differentially-expressed genes (> 2-fold change; omitting pseudogenes, noncoding genes, haemoglobin beta, and immunoglobulin kappa constant) were identified (Fig. 5B). Notably, PPP1R12B was significantly downregulated in CRC [31], and this protein is also involved in regulation of actin cytoskeleton, focal adhesion and cancer progression [32,33]. Considered that PEAK1 is a novel regulator of cytoskeletal organization, cell migration, and focal adhesion [8,9], and could potentially enhance PPP1R12B expression, we speculated that PEAK1 and PPP1R12B might play a synergistic role in CRC cell growth and metastasis suppression.

We verified that PEAK1 expression could markedly upregulate PPP1R12B in different CRC cell lines (Fig. 5C, Supplemental Figs. 5A and B). To further confirm this, we examined endogenous expression of PPP1R12B and PEAK1 in seven human CRC cell lines to explore their relationship. Results showed that PPP1R12B levels were positively correlated with PEAK1 expression in CRC cell lines (Fig. 5D, Supplemental Fig. 5C). We further showed that the expression of *PPP1R12B* mRNA was obviously decreased in 20 CRC tissues compared to that in corresponding non-cancerous tissues (Fig. 5E). Subsequently, we evaluated the association between *PPP1R12B* and *PEAK1* mRNA levels in 20 pairs of matched CRC tissues and adjacent normal tissues. qRT-PCR analysis indicated a significantly positive correlation between *PPP1R12B* and *PEAK1* mRNA levels in CRC tissues (Fig. 5F). Collectively, these results show that PEAK1 down-regulation might be the primary mechanism of impaired PPP1R12B expression in CRC.

3.6. PEAK1 attenuates the Grb2/PI3K/Akt pathway partly by regulating the Shc site in CRC cells

Modulation of PEAK1 protein levels was found to be associated with several known cytoskeletal regulatory proteins including p130Cas, Crk and ERK, suggesting that it might affect proliferation and migration through the p130Cas/Crk/Rac1 and Ras/Raf/ERK pathways [6,7]. In addition, PEAK1 is a scaffold that enables Shc1 to switch from Grb2-dependent mitogenic activity to Grb2-independent functions [7]. KEGG pathway analysis indicated that PEAK1 could potentially regulate ~11 pathways including the tumor-related PI3K/Akt, FoxO and insulin pathways (Supplemental Figs. 6A and B). Notably, the Grb2 scaffold protein, FoxO and insulin signalling pathways are known to be associated with the PI3K/Akt pathway during cancer progression [7,34]. Therefore, we simultaneously examined the activation of Rac1, ERK1/2, Grb2, PI3K and Akt in PEAK1-overexpressing CRC cells and corresponding control cells. We found that PEAK1 overexpression did not

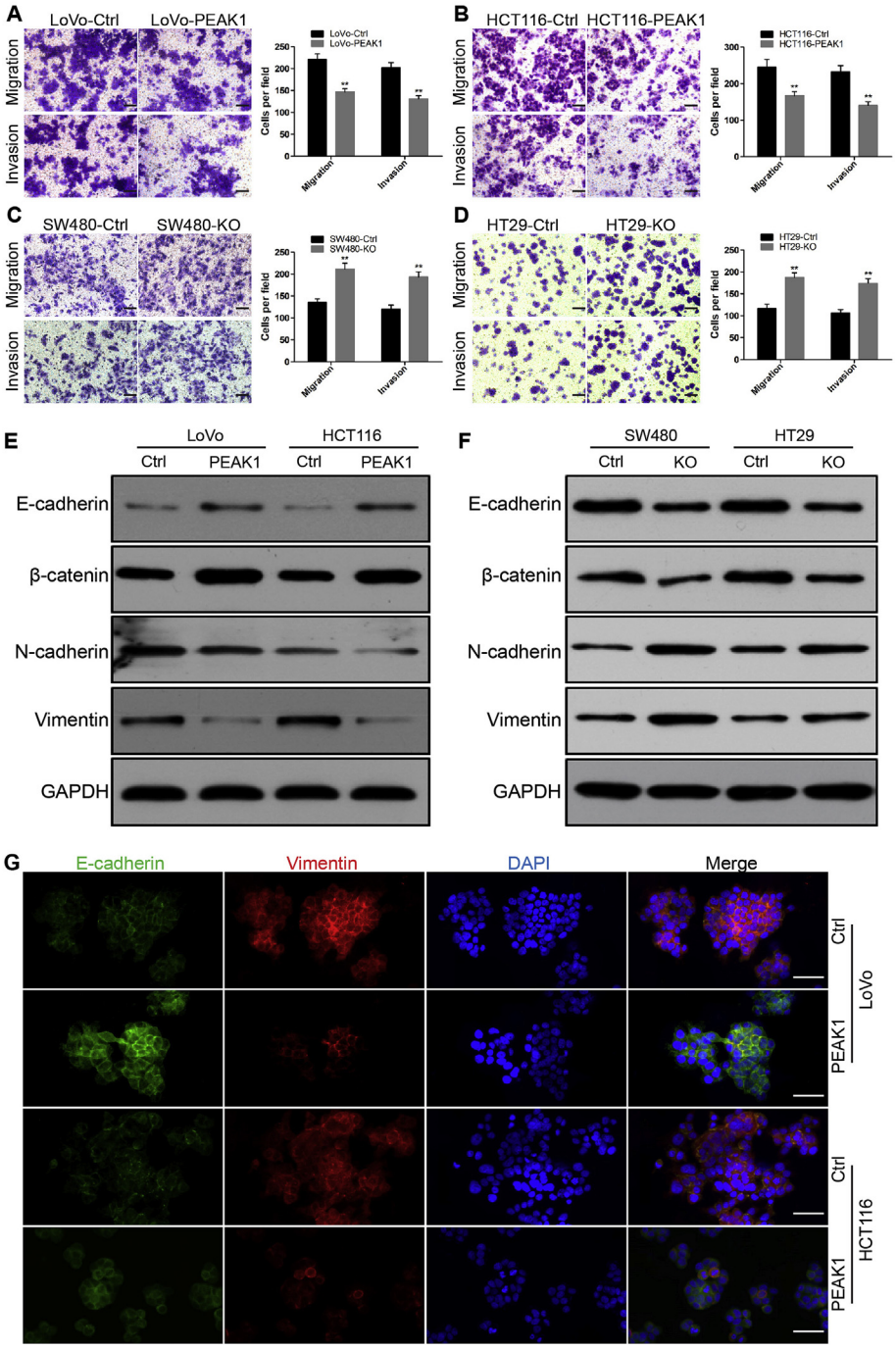

Fig. 3. PEAK1 inhibits cell migration, invasion, and EMT. (A, B) Migration and invasion assays of LoVo and HCT116 cells transfected with PEAK1-overexpression and negative control vectors. Scale bar, 100 μm. (C, D) Migration and invasion assays of SW480 and HT29 cells transfected with PEAK1 knockout lentiviral vectors and negative control vectors. Scale bar, 100 μm. (E) Upregulation of E-cadherin and β-catenin expression, and downregulation of N-cadherin and vimentin expression in LoVo-PEAK1 and HCT116-PEAK1 cells was assessed by western blot analysis. (F) PEAK1 knockout resulted in decreased expression of E-cadherin and β-catenin, and increased expression of N-cadherin and vimentin in SW480 and HT29 cells. (G) Immunofluorescent staining of E-cadherin and vimentin expression in PEAK1-overexpression LoVo and HCT116 cells or corresponding negative control cells. Scale bar, 150 μm. These data represent at least three independent experiments. Error bars show mean ± SD. $^{**}P < 0.01$.

markedly affect the phosphorylation of Rac1 and ERK1/2 (Supplemental Figs. 6C and D). However, as expected, Grb2, PI3K and Akt phosphorylation was significantly decreased in PEAK1-overexpressing cells (Fig. 6A, Supplemental Fig. 6E). In contrast, PEAK1 knockout obviously increased the activation of Grb2, PI3K and Akt in CRC cells (Fig. 6B, Supplemental Fig. 6F).

PEAK1 P1153 and Y1188 are necessary for Crk and Shc binding, respectively [6,7], which also can recruit or interact with Grb2 to regulate the transduction of PI3K/Akt signalling [35,36]. To further explore whether PEAK1 P1153 and Y1188 sites can regulate PI3K/Akt signalling via regulating the activation of Grb2 in CRC cells, we established PEAK1 wild-type (WT), Y1188 mutant (△Shc), P1153 mutant (△Crk) and negative control vectors for expression in LoVo cells. PEAK1 WT and △Crk both attenuated the phosphorylation of Grb2, PI3K and Akt compared to that with the vector control. Interestingly, PEAK1 △Shc mutant partially restored Grb2, PI3K and Akt activation compared to that with PEAK1 WT and △Crk vectors (Fig. 6C, Supplemental Fig. 7A). However, △Crk and △Shc mutants did not affect the expression of PPP1R12B compared to that with PEAK1 WT (Fig. 6C, Supplemental Figs. 7A and B). In addition, PEAK1 WT and △Crk markedly inhibited the tumor sphere-forming ability of LoVo cells compared to that with the vector control. PEAK1 △Shc failed to remarkably abrogate the suppressive role of PEAK1 on tumor sphere formation compared to that with PEAK1 WT and △Crk (Fig. 6D). We also compared the growth and invasion of PEAK1 WT- and mutant-expressing LoVo cells by performing clonogenic and transwell invasion assays. PEAK1 △Shc partially restored clonogenic and invasive abilities compared to those with PEAK1 WT and △Crk (Supplemental Figs. 7C and D). These data indicate that PEAK1 expression attenuates the Grb2/PI3K/Akt pathway partly through regulating Shc binding in

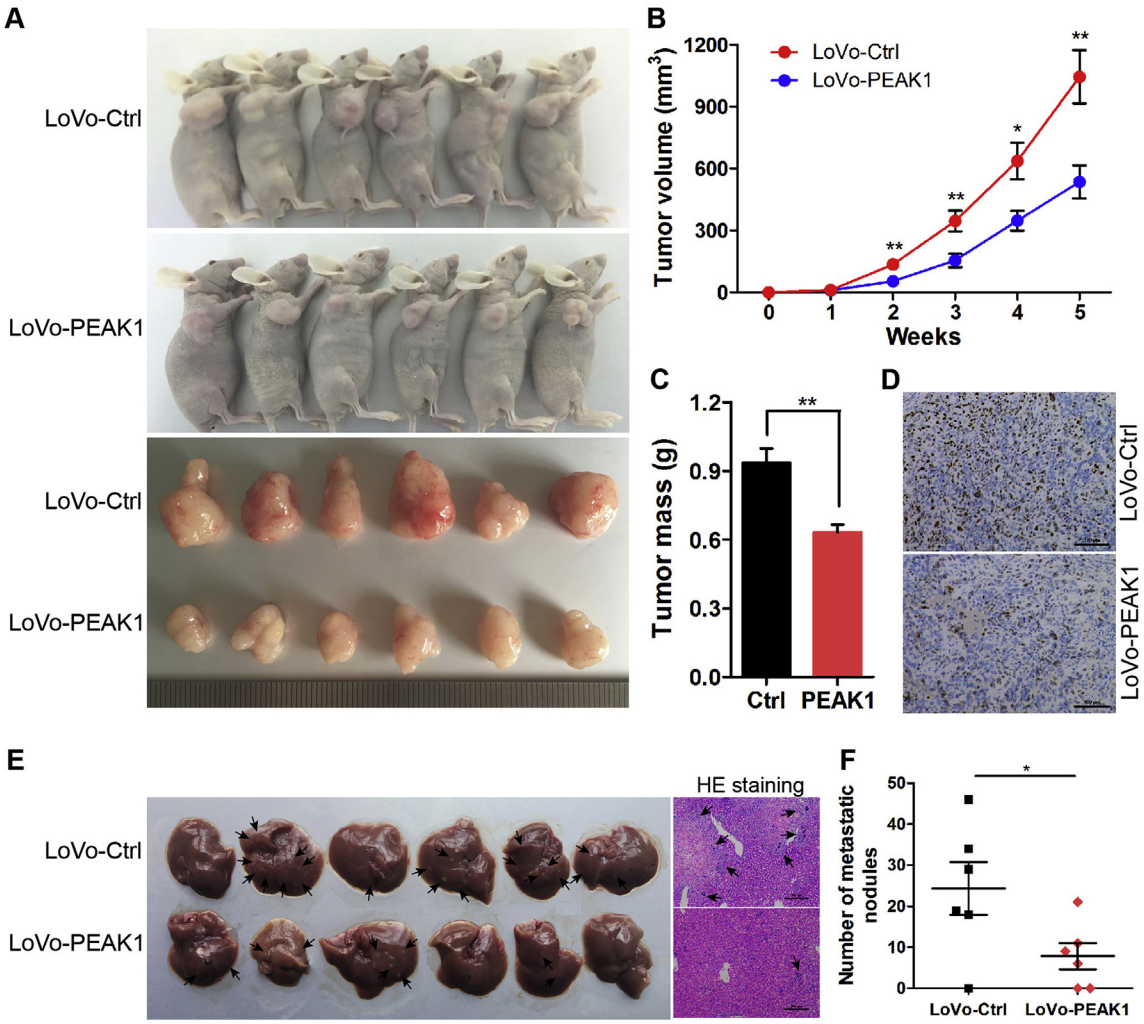

Fig. 4. PEAK1 suppresses tumorigenesis and metastasis in mice model. (A) Stable PEAK1-overexpression LoVo cells and control cells were inoculated into nude mice. These graphs show the tumor xenografts 5 weeks after ectopic-subcutaneous implantation in nude mice. (B–D) PEAK1 overexpressing cells of group exhibited attenuated tumor growth in nude mice compared with the control group. The effect of PEAK1 on tumor growth was evaluated based on tumor volumes, weights, and ki67 expression. Scale bar, 100 μm. (E) Images of mouse livers 35 days and representative pictures of liver metastasis by HE staining after tail vein injection of 1×10^6 PEAK1 overexpressing LoVo cells or negative control cells. Scale bar, 200 μm. (F) The numbers of liver metastatic colonies were counted and analysed with Student's t-test. Error bars show mean ± SD.*$P < 0.05$; **$P < 0.01$.

CRC cells.

3.7. PPP1R12B enhances the suppressive effects of PEAK1 on the biological behaviors of CRC cells and signal activation

Previous studies demonstrated that PPP1R12B is downregulated in CRC tissues and plays an important role in oncogenic signalling in breast cancer [31,33]. However, its functional in CRC progression was unknown. We constructed a PPP1R12B-overexpression vector (named p-PPP1R12B) and a corresponding control vector (named p-Cont), which were used to transiently transfect LoVo cells. We found that PPP1R12B overexpression markedly inhibited cell proliferation, migration, and invasion as compared to those in the control group (Supplemental Figs. 8A and B). Conversely, HT29 cell proliferation, migration and invasion were elevated with PPP1R12B siRNA (Supplemental Figs. 8C and D). In addition, PPP1R12B overexpression promoted the expression of PEAK1, p21 and E-cadherin, but reduced the expression of ki67 and N-cadherin in CRC cells, whereas PPP1R12B knockdown had the opposite effects (Supplemental Figs. 9A and B). Taken together, these data indicate that PPP1R12B might suppress CRC cell proliferation and invasion.

To address whether PPP1R12B expression mediates PEAK1-induced cell growth, metastasis, and Grb2/PI3K/Akt pathway suppression, we silenced its expression in PEAK1-overexpressing cells, or overexpressed it in PEAK1-knockout cells. We showed that PPP1R12B knockdown significantly decreased the suppressive effects of PEAK1 on Grb2/PI3K/Akt signalling and ki67 expression (Fig. 7A, Supplemental Fig. 10A). Functional studies suggested that the inhibitory effects of PEAK1 on cell proliferation, migration, and invasion were also rescued by silencing PPP1R12B expression (Fig. 7B and C). However, ectopic PPP1R12B expression in PEAK1-knockout HT29 cells resulted in the opposite effects on signalling activation, ki67 and E-cadherin expression, cells growth, and metastatic properties *in vitro* (Fig. 7D–F, Supplemental Fig. 10B). Then, we performed IHC to examine the expression of PPP1R12B, phosphor-PI3K, phosphor-Akt, phosphor-Grb2, ki67 and E-cadherin in human CRC tissues, to further determine their correlation with PEAK1 expression. In accordance with *in vitro* results, PEAK1 expression was inversely associated with phosphor-Grb2, phosphor-PI3K, phosphor-Akt and ki67 protein expression in 60 clinical CRC tissues. Additionally, the positive expression of PEAK1 was obviously correlated with high PPP1R12B and E-cadherin protein expression (Supplemental Figs. 11A and B). Collectively, these data indicate that the PEAK1–PPP1R12B axis attenuates Grb2/PI3K/Akt signalling, which then suppresses cell growth and metastasis in human CRC.

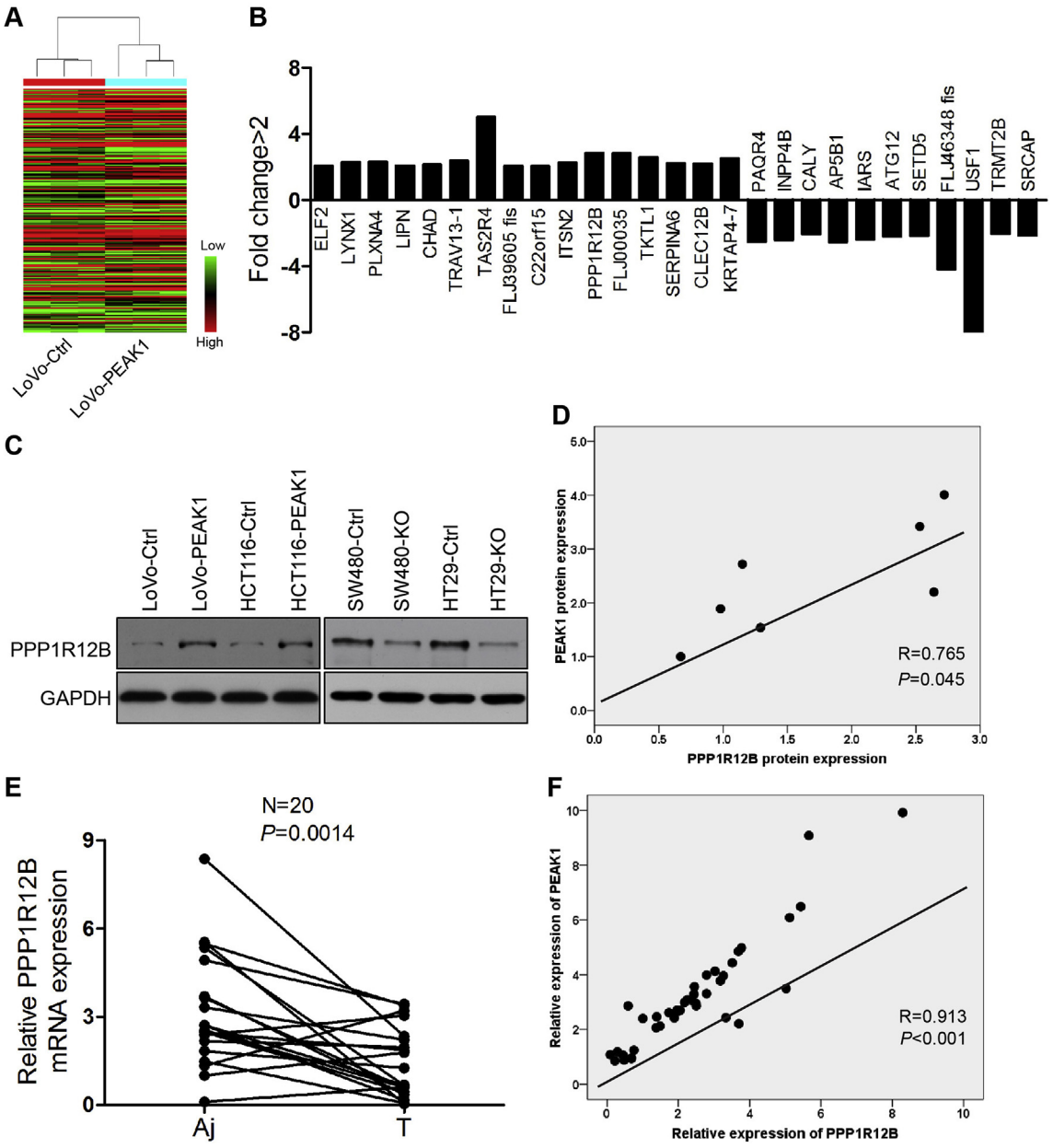

Fig. 5. PEAK1 positively regulates PPP1R12B expression in human CRC. (A) Heatmap of gene expression in PEAK1-overexpression LoVo cells and control cells. (B) 27 differentially expressed genes (> 2-fold change) were identified. (C) The levels of PPP1R12B protein expression in PEAK1-overexpression and knockout CRC cells were detected through western blot analysis. (D) A positive relationship between PPP1R12B and PEAK1 protein expression was demonstrated in seven human CRC cell lines based on Spearman's correlation. (E) *PPP1R12B* mRNA expression in 20 paired CRC tissues and adjacent normal tissues was assessed by qRT-PCR analysis. GAPDH was used as an internal control. Aj, adjacent normal tissue; T, CRC tissue. Error bars show mean ± SD. The *P* value is for paired *t*-test. (F) Scatterplots showing the positive linear correlation between *PPP1R12B* and *PEAK1* mRNA expression in 20 paired CRC tissues and adjacent normal tissues (total of 40 samples). Data represent three independent experiments.

3.8. PI3K and Akt inhibitors recapitulate impaired PEAK1 function with respect to CRC cell proliferation and invasion

As shown, PEAK1 knockout significantly increased PI3K and Akt phosphorylation in SW480 and HT29 cells (Fig. 6B, Supplemental Fig. 6F). Recent findings indicated that small molecule inhibitors of PI3K/Akt signalling can be applied for tumor treatment [37,38]. Notably, it is well demonstrated that PI3K/Akt signalling is aberrantly activated in CRC cells, and the use of their inhibitors decrease CRC cell growth and invasion [39,40]. To explore a potential therapeutic strategy for CRC patients with impaired PEAK1 expression, we determined whether the PI3K inhibitor LY294002 and the Akt inhibitor ipatasertib could inhibit PEAK1 knockout-induced CRC cell proliferation, migration, and invasion. As shown in Fig. 8A, LY294002 or ipatasertib significantly reduced the proliferation of PEAK1-knockout SW480 cells as compared to that in the control group. Similar data were found for PEAK1-knockout HT29 cells (Fig. 8B). In addition, the migration and invasion of PEAK1-knockout SW480 and HT29 cells were markedly inhibited by LY294002 or ipatasertib (Fig. 8C–F). These results suggest that inactivation of PI3K/Akt signalling plays a critical role in mediating PEAK1-induced suppression of CRC growth and metastatic properties.

4. Discussion

Non-receptor PTKs (NRTKs) are important components of signalling

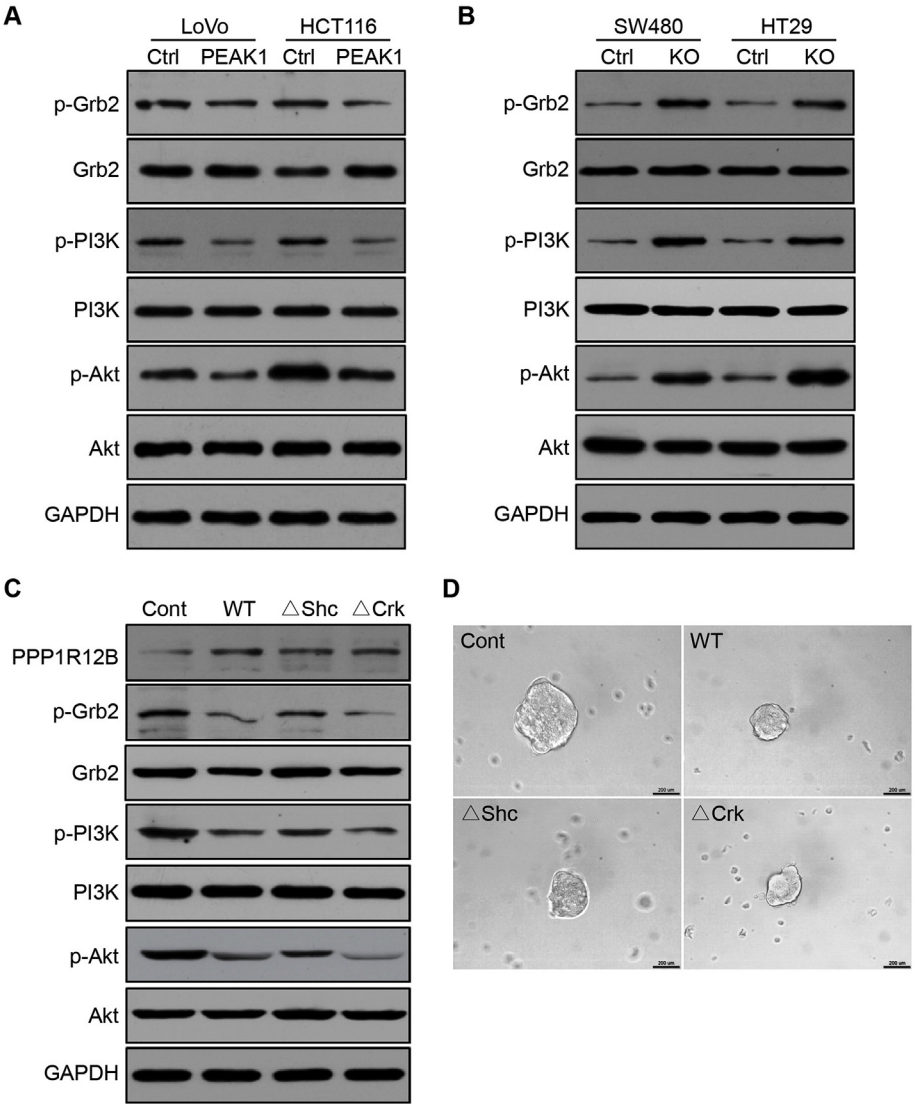

Fig. 6. PEAK1 attenuates the Grb2/PI3K/Akt pathway partly through regulating Shc site in CRC cells. (A) PEAK1 overexpression reduces the activation of Grb2, PI3K and Akt in LoVo and HCT116 cells. (B) Western blot analysis of phospho-Grb2, phospho-PI3K and phospho-Akt in PEAK1 knockout SW480 and HT29 cells as well as corresponding control cells. (C) The activation of Grb2/PI3K/Akt signalling pathway and the expression of PPP1R12B protein in LoVo stably expressing vector control (Cont), PEAK1 wild-type (WT), Y1188 mutant (△Shc, cannot bind Shc), P1153 mutant (△Crk, cannot bind Crk) was detected by western blot analysis. (D) Representative phase-contrast images of vector control, PEAK1-WT, PEAK1-△Shc and PEAK1-△Crk tumor spheres at 15 days. Data represent three independent experiments. Scale bar, 200 μm.

pathways that regulate fundamental cellular functions such as survival, apoptosis, proliferation, and migration [41]. Dysregulation of NRTKs and/or NRTK-mediated signalling has been implicated in malignant transformation and carcinogenesis, which has emerged as primary targets for cancer therapy [41,42]. In this study, we showed that the NRTK PEAK1 is downregulated in CRC tissues, and related to tumor size and differentiation status, cancer metastasis, and tumor grade. Overexpression of PEAK1 suppressed CRC cell growth and metastasis *in vitro* and *in vivo* through upregulating PPP1R12B, which then inhibited Grb2/PI3K/Akt signalling. PEAK1 knockout had the opposite effects on gene expression, cancer cell behaviours, and signal transduction. Moreover, PPP1R12B knockdown reversed the effects of PEAK1 overexpression, whereas enforced expression recapitulated the suppressive effects of PEAK1. Altogether, our results suggest that the PEAK1–PPP1R12B axis is a potential tumor-suppressive loop in human CRC.

DNA amplification or chromosomal loss is a common mechanism leading to oncogenic activation during tumorigenesis. *PEAK1* is located on chromosomal band 15q24.3 in humans, which is commonly lost in CRC [43,44]. Several studies have reported that PEAK1 is amplified in many types of human malignancies including colon cancer [9], pancreatic carcinoma [10], breast cancer [13], and lung cancer [45]. In contrast, Qingqu et al. identified downregulated PEAK1 expression in gastric cancer, which was significantly associated with poor prognosis [16]. Interestingly, our results also indicated that PEAK1 was reduced in CRC tissues, and obviously correlated with cancer metastasis and clinical stage. Although the reported expression patterns of PEAK1 in various cancers remain controversial, our data suggests that impaired PEAK1 expression might play an important role in CRC pathogenesis and progression. However, the precise clinical value of PEAK1 expression remains to be fully elucidated.

Eukaryotic translation initiation factor 5A–PEAK1 signalling contributes to the development and pathogenesis of pancreatic cancer [11,12]. In addition, oncogenic KRas induces a PEAK1-dependent kinase amplification loop to drive pancreatic cancer metastatic growth and therapy resistance [10]. In breast cancer, PEAK1 was identified as a key molecular switch in TGFβ-induced EMT and tumor metastasis [13,14]. Our previous work indicated that PEAK1 promoted lung cancer metastasis and EMT by regulation of ERK1/2 and Janus kinase-2 signalling [45]. A recent study shown that PEAK1 might be act as a tumor promoter in CRC, which is regulated by the epidermal growth factor receptor (EGFR)/KRas signalling axis and miR-181d [46]. Collectively, these studies suggest that PEAK1 might act as an oncogenic kinase in these cancers. Additionally, PEAK1 is required for angiogenesis during vertebrate development and cancer by integrating cell adhesion and growth factor cues from the extracellular environment [47]. However, we provide surprising evidence that PEAK1 expression inhibits CRC growth and metastasis *in vitro* and *in vivo*. Moreover, PEAK1

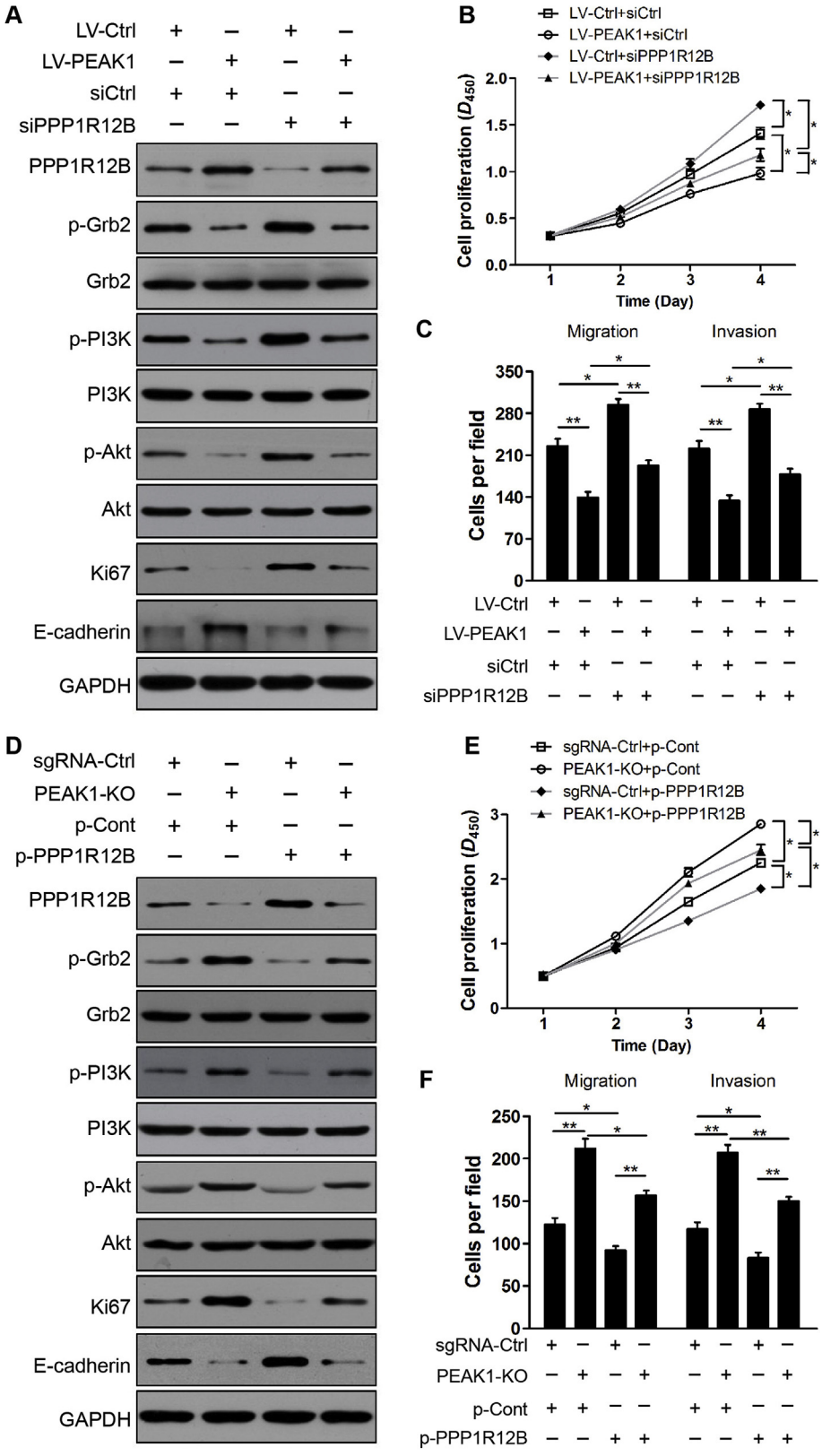

Fig. 7. PPP1R12B mediates PEAK1-induced cell growth, metastasis, and Grb2/PI3K/Akt pathway suppression. (A) Expression of PPP1R12B, phospho-Grb2, phospho-PI3K, phospho-Akt, ki67 and E-cadherin was determined by western blot analysis in stable PEAK1-overexpression LoVo cells and control cells transfected with PPP1R12B siRNA or negative control siRNA. (B) CCK-8 assay of stable PEAK1-overexpression LoVo cells and control cells. Cells were treated as in (A). (C) Migration and invasion assay of stable PEAK1-overexpression LoVo cells and control cells. Cells were treated as in (A). (D) Expression of PPP1R12B, phospho-Grb2, phospho-PI3K, phospho-Akt, ki67 and E-cadherin was determined by western blot analysis in PEAK1 knockout HT29 cells and control cells transfected with p-PPP1R12B or p-Cont plasmid. (E) CCK-8 assay of PEAK1 knockout HT29 cells and control cells. Cells were treated as in (D). (F) Migration and invasion assay of PEAK1 knockout HT29 cells and control cells. Cells were treated as in (D). These data represent at least three independent experiments. Error bars show mean ± SD. $^*P < 0.05$.

was found to be important for suppressing EMT. Therefore, our data show that PEAK1 acts as a tumor suppressor for CRC. Obviously, these results contradict the previous findings by Lanlan et al. [46]; we speculate that PEAK1 might have specific roles based on the CRC population. Further, the previous study explored the effect of PEAK1 on CRC cell biological behaviours, based on the activation of EGFR/KRas and KRas-induced PEAK1 expression, suggesting that EGFR/KRas signalling was required for PEAK1-mediated CRC. However, we used stable PEAK1-overexpressing and knockout cell lines to detect the function of PEAK1-itself. These are two completely different concepts.

Recent studies demonstrated that PEAK1, as a separate signalling entity, is involved in signal output and biological response in cancer

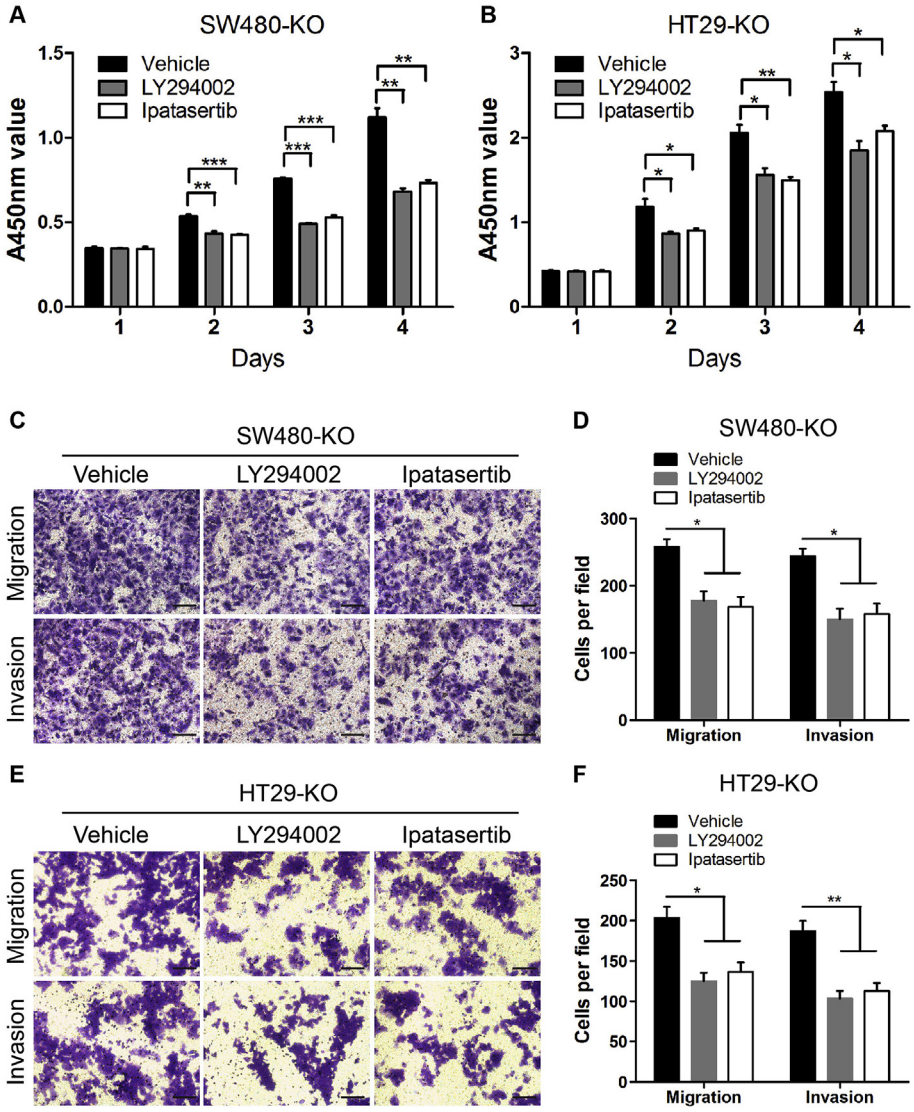

Fig. 8. Inhibitors of PI3K and Akt reduce impaired PEAK1 effects on CRC cell proliferation, migration, and invasion. (A, B) PEAK1 knockout SW480 or HT29 cells were subjected to CCK-8 assays in the absence (vehicle) or presence of 5 μM LY294002 or ipatasertib, respectively. (C, D) PEAK1 knockout SW480 cells were subjected to transwell migration and invasion (for 24 h) assays in the absence (vehicle) or presence of 5 μM LY294002 or ipatasertib, respectively. Migration and invasion of SW480-KO cells were quantitatively analysed. (E, F) LY294002 or ipatasertib could markedly decrease the migration and invasion of HT29-KO cells (two clones, C and D). These data represent at least three independent experiments. Scale bar, 100 μm. Error bars show mean ± SD. $^{*}P < 0.05$; $^{**}P < 0.01$; $^{***}P < 0.001$.

pathogenesis [48,49]. However, the potential targets and molecular mechanism of PEAK1 in tumorigenesis are still limited. We found that PEAK1 potentially enhances PPP1R12B expression and downregulates PI3K/Akt signalling. To further confirm the relationship between PPP1R12B and PEAK1, we compared their expression patterns in CRC cell lines and clinical samples. Indeed, PEAK1 was identified as a positive regulator of PPP1R12B in human CRC. It is well known that dysregulation and/or activation of PI3K/Akt signalling is associated with cancer progression and treatment-resistance [50,51]. In our ongoing studies, we identified that PEAK1 expression significantly suppresses the Grb2/PI3K/Akt pathway in CRC cells (Fig. 6A and B). Shc and Crk adaptor proteins regulate cell proliferation, apoptosis and migration during tumor development through participating in several cellular signalling networks [52,53]. Interestingly, PEAK1 △Shc, but not △Crk, partly restored Grb2, PI3K and Akt activation, tumor sphere formation, cells growth, and invasion. Notably, △Shc and △Crk mutants did not affect PPP1R12B expression, indicating that Shc-binding site might be only one functional residue used by PEAK1 to inhibit biological behaviours and signal transduction in CRC.

To clarify whether PEAK1 inhibits CRC growth and metastasis through PPP1R12B-mediated inactivation of Grb2/PI3K/Akt signalling, we first analysed the functional role of PPP1R12B in CRC cells. Our results suggested that PPP1R12B expression mediates the suppressive effect of PEAK1 on cell growth, metastasis, and Grb2/PI3K/Akt signalling in human CRC. Moreover, the expression of PPP1R12B and E-cadherin were positively associated with PEAK1 expression, but the expression of phosphor-Grb2, phosphor-PI3K, phosphor-Akt and ki67 were inversely correlated with PEAK1 expression in human CRC tissues. Of note, small molecule antagonists of PI3K/Akt signalling are being tested for CRC therapies [54,55]. Here, we found that LY294002 or ipatasertib could block PEAK1 loss-of-function-mediated CRC cell proliferation and invasion. Thus, drugs targeting PI3K/Akt signalling might be potentially useful to treat CRC with impaired PEAK1–PPP1R12B axis in combination with standard chemotherapy.

In conclusions, our data provide, for the first time, important insights into the tumor suppressive role of PEAK1 in CRC. We demonstrated that PEAK1 inhibits tumor growth and metastasis via upregulating PPP1R12B, which then attenuates Grb2/PI3K/Akt signalling. Moreover, PEAK1 expression was inversely correlated with tumor size, cancer metastasis, clinical grade, and Grb2/PI3K/Akt activation in CRC tissues. These results collectively show that the PEAK1–PPP1R12B axis is a promising anti-cancer therapeutic target.

Conflicts of interest

All patients provided written informed consent before surgery, and our study were approved by the Ethics Committee of the Affiliated Zhongda Hospital of Southeast University, and the Affiliated Hospital of

Zunyi Medical University according to the 1975 Declaration of Helsinki. In addition, I would like to declare on behalf of my co-authors that we have no competing interests.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.canlet.2018.11.014.

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