Low expression of PEBP1P2 promotes metastasis of clear cell renal cell carcinoma by post-transcriptional regulation of PEBP1 and KLF13 mRNA

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Abstract
Background: Pseudogenes play an essential role in tumor occurrence and progression. However, the functions and mechanisms of pseudogenes in clear cell renal cell carcinoma (ccRCC) remain largely elusive.

Methods: We quantified PEBP1P2 expression in ccRCC tissues and cells using fluorescence in situ hybridization and real-time PCR. Besides, we evaluated the role of PEBP1P2 in ccRCC using a lung metastasis model and a transwell assay. Finally, we documented the interactions between PEBP1P2, PEBP1, and KLF13 by performing luciferase, RNA immunoprecipitation, RNA pulldown, and targeted RNA demethylation assays.

Results: Low PEBP1P2 expression correlates significantly with advanced stages and poor prognosis in ccRCC patients. Besides, PEBP1P2 overexpression inhibits ccRCC metastasis formation in vivo and in vitro. Interestingly, PEBP1P2 directly interacted with 5-methylcytosine (m5C)-containing PEBP1 mRNA and recruited the YBX1/ELAVL1 complex, stabilizing PEBP1 mRNA. In addition, PEBP1P2 increased KLF13 mRNA levels by acting as a sponge for miR-296, miR-616, and miR-3194.

Conclusions: PEBP1P2 inhibits ccRCC metastasis formation and regulates both PEBP1 and KLF13. Therefore, molecular therapies targeting PEBP1P2 might be an effective treatment strategy against ccRCC and other cancers with low PEBP1P2 levels.

Keywords: PEBP1P2, m5C modification, Clear cell renal cell carcinoma, PEBP1, KLF13

Background
The most dreadful kidney tumor, renal cell carcinoma (RCC), accounts for 4% of all cancer cases worldwide [1–3]. In recent years, smoking, alcohol consumption, and obesity have increased RCC incidence. The most common form of RCC is clear cell renal cell carcinoma (ccRCC), representing 70–80% of cases [4]. In solid ccRCC tumors, the angiogenesis-related signaling pathway is activated, making them highly vascularized and setting up favorable conditions for metastasis formation [5]. Around 25–30% of patients diagnosed with...
cancer have metastatic or regionally advanced tumors and, in 2% of those who undergo resection, cancer recurs [6]. Because of the low sensitivity of metastatic RCC to chemotherapy and radiation, patients with metastatic RCC (mRCC) have poor outcomes. Besides, sorafenib, one of the first-line drugs against ccRCC, is less potent on mRCC [7, 8]. Therefore, it is crucial to find new therapeutic strategies against ccRCC and mRCC.

Pseudogenes accumulate evolutionary mutations [9, 10] but have no protein-coding capacity, although many are transcriptionally active [11]. The ENCODE project identified around 15,000 pseudogenes in the human genome [12]. Pseudogenes are useful to investigate the course of evolution and contain precious clues about genome dynamics [13]. Recently, a deeper understanding of the biological function of non-coding RNAs in human malignancies has emerged, and this category of natural non-coding RNAs is gathering interest [14, 15]. A growing body of literature highlights the critical roles of pseudogenes in cancers [11]. However, only a few studies based on bioinformatic analysis indicate that pseudogenes could function as essential mediators in ccRCC. These results highlight the urgency of documenting the relationship between pseudogenes and metastatic ccRCC.

Phosphatidylethanolamine binding protein 1 pseudogene 2 (PEBP1P2) can be transcribed into a 409 nucleotide-long non-coding RNA strand previously named IncRNA5 [16]. PEBP1P2 inhibits abnormal proliferation, migration, and phenotypic switching in vascular smooth muscle cells during cardiovascular diseases [16]. As an important ferroptosis regulator, PEBP1 mediates many tumor processes, including development, metastasis formation [17], and tumor microenvironment [18], for example through inflammation [19]. However, while the correlation between PEBP1 and tumors has been proven, the role of PEBP1P2 in ccRCC remains unclear.

Our results show that PEBP1P2 (which is downregulated by STAT4) inhibits migration, invasion, and metastasis formation. Mechanistic studies revealed that PEBP1P2 prevents PEBP1 mRNA decay through direct interaction and mediates KLF13 expression via post-transcriptional regulation. Thus, this work reveals a novel regulatory mechanism involving PEBP1P2, a critical regulatory factor in ccRCC metastases.

Materials and methods
Cell culture and tissue samples
The Chinese Academy of Sciences (Shanghai, China) provided the HEK293T, HK-2, ACHN, A-498, and 786-O cells. We cultured the cells at 37 °C, under a 5% CO₂ atmosphere, in Dulbecco’s modified Eagle medium (DMEM) with high glucose contents, 10% fetal bovine serum (FBS, Gibco, Grand Island, NY), and 1% penicillin–streptomycin (Gibco). A senior pathologist checked the patient samples collected by the Nanjing Drum Tower Hospital (Department of Pathology, Nanjing Drum Tower Hospital). Each patient provided informed consent for the use of their tissues in scientific research.

RNA isolation and quantitative real-time PCR assays
We extracted total RNA using RNA-easy Isolation Reagent (Vazyme Biotech Co., Ltd., Nanjing, China) following the manufacturer’s instructions. Next, we reverse-transcribed RNA into complementary DNA (cDNA) and quantified it by quantitative real-time PCR (qRT-PCR) using the Vazyme HiScript II Q Select RT SuperMix for qPCR and ChamQ Universal SYBR qPCR Master Mix, respectively. We normalized quantification values using 18 s rRNA as an internal reference. We then quantified microRNA (miRNA) expression using a miRNA 1st Strand cDNA Synthesis Kit (by stem-loop) and miRNA Universal SYBR qPCR Master Mix (Vazyme). The loading control was set to U6 snRNA. Additional file 1: Table S1 lists the RNA primers.

ChIP assay and dCas9-ChIP assay
We assessed the interaction between STAT4 and the PEBP1P2 promoter by performing ChIP and dCas9-ChIP experiments with a ChIP Kit (Bersin Biotechnology Co., Ltd., Guangzhou, China) according to the manufacturer’s instructions. Briefly, we fixed and lysed the cells, then sonicated genomic DNA to ~ 200 bp fragments. Next, we pre-treated precipitated chromatin overnight at 4 °C with STAT4 antibody or IgG. Afterward, we purified the immunoprecipitated chromatin and analyzed it using qRT-PCR. We then transfected 786-O cells with Flag-labeled dCas9 and guide RNA targeted to the PEBP1P2 promoter to conduct the dCas9-ChIP assay. Anti-Flag enriched the dCas9 complex containing PEBP1P2 promoter fragments, while dCas9 enriched the proteins binding to the PEBP1P2 promoter. Additional file 1: Table S2 lists the primers targeting potential STAT4 binding sites in PEBP1P2 promoters.

Dual-luciferase reporter assay
We amplified the potential STAT4-binding region of the PEBP1P2 promoter and placed it in a pGL3-Basic vector. We also ligated the regions containing a potential miRNA response element in the 3’-UTR of KLF13 mRNA to the pGLO-miR vector. Next, we transfected HEK293T cells with the indicated luciferase reporter plasmid, pRL-TK, and appropriate plasmids. Finally, we quantified the activities of the Firefly and Renilla luciferases using a Dual-Luciferase Reporter Kit (Vazyme) and normalized
the Firefly luciferase activity against that of Renilla luciferase.

**RNA immunoprecipitation (RIP) and MS2-RIP assay**

We performed the RIP assays using a RIP kit (Bersin) according to the manufacturer’s instructions. We lysed the cells using the RIP lysis buffer containing protease and RNase. After centrifuging the lysate, we incubated the supernatant with specific antibody-conjugated beads overnight at 4 °C. We then thoroughly washed, eluted, and purified the binding complexes. Next, we reverse-transcribed RNA into cDNA to perform qRT-PCR. We extended the PEBP1P2 with a 12× MS2 stem-loop for the MS2-RIP assay. We then co-transfected cells with the MS2-GFP vector and PEBP1P2 with a MS2 stem-loop and lysed them in RIP lysis buffer. Then, we repeated the steps as in RIP assay and analyzed the protein complex by Western blot. We also evaluated the modification levels of the target mRNA using and MeRIP kit (Bersin) per the manufacturer’s instructions. Finally, we analyzed the binding complexes by qRT-PCR.

**RNA pulldown assays**

We conducted the pulldown assays with biotinylated PEBP1P2 probes (Bersin). Briefly, we produced probe-coated beads by incubating beads with the probe. After lysing and centrifuging the cells, we incubated the clarified cell lysates at 4 °C overnight with a probe-coated bead mixture. After washing and purifying the RNA complexes, we analyzed RNA by qRT-PCR and proteins by Western blot.

**Western blot**

First, we lysed the cells with RIPA lysis buffer (Beyotime, Shanghai, China). After centrifugation, we mixed the separated soluble fraction with 5× loading buffer and heated it. We then separated total protein samples by sodium dodecyl sulfate–polyacrylamide gel electrophoresis and transferred them to polyvinylidene difluoride membranes (Millipore, Darmstadt, Germany). We blocked the membranes with 5% nonfat milk for 1 h at room temperature. Then, we incubated them with the primary antibodies in 3% bovine serum albumin overnight at 4 °C. Next, we incubated the membranes with the secondary antibodies and HRP-conjugated antigens at room temperature for 1 h. Finally, we revealed the bands with the chemiluminescent ECL reagent (Vazyme) and analyzed them using ImageJ software (National Institutes of Health), using ACTB as the internal control.

**Fluorescence in situ hybridization (FISH)**

Servicebio synthesized FAM-labeled PEBP1P2 probes. We performed the FISH assay using a FISH kit (Servicebio) per the manufacturer’s instructions. After DAPI staining, we captured images with a Nikon DS-U3.

**Immunohistochemistry**

We deparaffinized, rehydrated, and incubated paraﬁn-embedded sections with 3% hydrogen peroxide for 3 min. Next, we permeabilized them using phosphate-buffered saline with 0.3% Triton for 15 min, then blocked them with 3% bovine serum albumin solution for 1 h. We then incubated the sections with the primary antibody overnight at 4 °C and with the secondary antibody for 1 h at room temperature. Next, we washed the sections three times with phosphate-buffered saline with Tween 20 and visualized the antibody using a DAB chromogenic kit (Servicebio, Wuhan, China) according to the manufacturer’s instruction and counterstained it with hematoxylin.

**Transwell assay**

We assessed the migration and invasion of tumor cells using a transwell assay. For migration, we used uncoated polycarbonate inserts (Millipore), while we used Biocoat™ inserts (BD Biosciences) for invasion. After starving the cells overnight, we filled the upper chamber with 1–5 × 10⁴ cells suspended in DMEM without FBS and the lower chamber with 500 μL of DMEM containing 10% FBS. After crystal violet staining, we counted and analyzed the positive cells under a microscope.

**Targeted RNA demethylation system**

We constructed a targeted RNA demethylation system via standard procedures such as enzyme digestion, PCR, and subcloning, as previously described [20, 21]. In brief, we fused the full-length TET1, TET2, or ALKBH5 to dCas13b and added NES to control the subcellular localization of dCas13b fusions. To target PEBP1 mRNA, we designed guide RNAs (gRNAs). Next, we co-transfected target cells with the dCas13b fusions and gRNAs.

**Plasmid construction, short hairpin RNA (shRNA), antisense oligonucleotides, lentivirus, and cell transfection**

We subcloned the PEBP1P2 sequence into a pCDH vector and fused 12× MS2 in this plasmid to perform an MS2-RIP assay. We purchased short hairpin RNA (shRNA) and lentivirus from OBIO Technology (Shanghai, China). Following the manufacturer’s instructions, we transfected the cells with the plasmids using Lipofectamine 3.0 (Hanbio, Shanghai, China). The sequences are provided in Additional file 1: Tables S4 and S5.

**Animal experiment**

We maintained 6-week-old NCG (NOD/ShiLtJ/Gpt-Prkdc<sup>em26Cd52</sup>Il2rg<sup>em26Cd23</sup>/Gpt) mice under specific
pathogen-free conditions. The mice received an injection of $5 \times 10^4$ cells via the tail vein to establish the lung metastasis model. After two months, we counted nodules on the lung surface with the naked eye and considered them as metastases. The Animal Care and Use Committee of Nanjing University approved all the procedures (Protocol Number IACUC-D2202057).

**Statistical analysis**
We analyzed continuous and categorical variables using the Mann–Whitney U test and the $\chi^2$ test, respectively. We compared values using Student’s t-test and one-way analysis of variance (ANOVA). We performed the statistical analyses with SPSS 22.0 (SPSS Inc., Chicago, IL) and plotted data with GraphPad Prism 8.0 (GraphPad Software, San Diego, CA). We considered that $P < 0.05$ indicated statistical significance (*$P < 0.05$, **$P < 0.01$, and *** $P < 0.001$). All values are expressed as the mean ± standard deviation.

**Results**

**Low PEBP1P2 expression levels contribute to metastasis formation in ccRCC**
To identify genes essential for ccRCC metastasis formation, we analyzed gene expression and prognostic data from the ccRCC dataset from The Cancer Genome Atlas (TCGA) using Gene Expression Profiling Interactive Analysis (GEPIA, http://geopia.cancer-pku.cn/) [22]. We found that a pseudogene, PEBP1P2, was the most important prognostic factor in ccRCC (Additional file 1: Table S1). Therefore, we evaluated the expression of PEBP1P2 in 21 pairs of ccRCC tissues and adjacent non-cancerous tissues by qRT-PCR. We found that 17 out of 21 (80.9%) cancerous specimens had lower PEBP1P2 RNA levels than the adjacent non-cancerous tissues (Fig. 1a and b). Analyzing PEBP1P2 expression in the ccRCC data from TCGA and evaluating the FISH assays results on 36 paired resected specimens confirmed this finding (Fig. 1c–f).

Besides, PEBP1P2 RNA levels were significantly correlated with advanced T stage, M stage, pathologic stage, and tumor progression (Additional file 1: Fig. S1, Table S2). Moreover, ccRCC patients with low PEBP1P2 levels had shorter overall survival, progress-free interval, and disease-specific survival than papillary RCC and chromophobe RCC patients (Fig. 1g–i, Additional file 1: Fig. S2). Next, we determined the diagnostic utility of PEBP1P2 by performing a receiver operating characteristic (ROC) curve analysis on the ccRCC datasets. We obtained a value of 0.835 with a 95% confidence interval of 0.784–0.887 (Fig. 1j), indicating that a low PEBP1P2 expression was correlated with poor prognosis in ccRCC patients.

To uncover the role of PEBP1P2 deficiency in ccRCC metastasis, we silenced PEBP1P2 in cells using shRNAs or antisense oligonucleotides, and overexpressed it using a Synergistic activation mediator (SAM) system or lentivirus vectors (Additional file 1: Fig. S3). Knocking down PEBP1P2 markedly enhanced cell migration and invasion. Correspondingly, upregulating PEBP1P2 notably reduced cell migration and invasion (Additional file 1: Fig. S4). In addition to the transwell assay, we established an orthotopic murine breast cancer model with an experimental tail-vein metastasis model to evaluate the role of PEBP1P2 on metastasis formation in vivo. Five weeks after the tail vein injection, mice with tumors expressing PEBP1P2 had significantly fewer metastatic nodules (Fig. 1k–m).

To confirm that PEBP1P2 participated in ccRCC metastasis, we selected 786-O cells with high migratory capacity (786-OHiMi) and low migratory capacity (786-OLoMi) by performing ten rounds of transwell migration (Additional file 1: Fig. S5a). This construction method has the advantage of producing two cell lines with similar genetic backgrounds. 786-OLoMi cells had higher PEBP1P2 RNA levels than 786-OHiMi cells (Additional file 1: Fig. S5b). Consistently, downregulating PEBP1P2 in 786-OLoMi cells promoted migration and invasion, and vice versa (Additional file 1: Fig. S5c). These results suggest that low PEBP1P2 expression levels contribute to metastasis formation in ccRCC.

**STAT4 reduces PEBP1P2 expression**
Using the PROMO website (http://alggen.lsi.upc.es/cgi-bin/promo_v3/promo/promoinit.cgi?dirDB=TF_8.3), we analyzed the sequence of the PEBP1P2 promoter region to investigate the upstream regulation of PEBP1P2 [23, 24]. We thus found 21 transcription factors potentially interacting with the promoter region of PEBP1P2. Next, we set the differential expression as the condition (Additional file 1: Fig. S6) and screened ten genes for further investigation. Subsequently, we treated 786-O cells with lentiviruses containing target-oriented shRNA or control shRNA. The changes in PEBP1P2 levels indicated that STAT4 and FOXP3 might be the upstream regulators of PEBP1P2 (Fig. 2a, Additional file 1: Fig. S7a–c). Afterward, we cloned the PEBP1P2 promoter region into a luciferase reporter plasmid and co-transfected HEK293T with it and shRNA. The dual-luciferase assays revealed that silencing STAT4 upregulated luciferase activity the most notably (Fig. 2a). Consistently, overexpressing STAT4, but not FOXP3, reduced the luciferase activity (Fig. 2c, Additional file 1: Fig. S7d). Finally, the ChIP assays confirmed that STAT4 directly bound the PEBP1P2 promoter region (Fig. 2d). Besides, the dCas9-ChIP assay revealed that STAT4, but not FOXP3,
Fig. 1 Low expression of pseudogene PEBP1P2 contributes to metastasis of ccRCC. a, b Real-time PCR analysis of PEBP1P2 RNA levels was conducted on 21 pairs of ccRCC tissues and corresponding non-cancerous tissues (a), and relative RNA level of PEBP1P2 was normalized to internal control 18 s rRNA (b). c, d The RNA level of PEBP1P2 was measured according to all samples (c) and the paired samples (d) in the ccRCC dataset TCGA database. e, f FISH detection of PEBP1P2 (green) RNA in ccRCC tissues and its adjacent non-cancerous tissues was performed, and nuclei were visualized with DAPI counterstaining (blue). The relative fluorescence intensity was shown as a scatter plot (e). g–i Kaplan–Meier curve was conducted to estimate overall survival (g), progress-free interval (h) and disease-specific survival (i), with a 95% confidence interval (dashed lines). j Receiver operating characteristic (ROC) analysis was constructed for quantifying response prediction. k, l Gross view of lung samples containing metastatic nodules (cyan arrows, metastatic nodules). m Slides of lungs stained with hematoxylin and eosin. m Number of the metastatic pulmonary lesions. The data are presented as the mean ± SD, **p < 0.01, ***p < 0.001
interacted directly with the PEBP1P2 promoter region (Fig. 2e).

Based on TCGA ccRCC dataset, tumor tissues had much higher STAT4 mRNA levels than normal (Fig. 2f and g), which was further confirmed by the data from the 21 paired resected samples (Fig. 2h). Similarly, these two datasets confirmed the negative correlation between STAT4 mRNA levels and PEBP1P2 RNA levels (Additional file 1: Fig. S7e and f). We also found that tumors expressed higher STAT4 levels than adjacent tissues in the Office of Cancer Clinical Proteomics Research (CPTAC) database and through immunohistochemistry staining (Fig. 2i–k). Moreover, silencing PEBP1P2 rescued the inhibition of migration and invasion induced by downregulating STAT4, and overexpressing PEBP1P2 might weaken the facilitation from the ectopic expression of STAT4 (Additional file 1: Fig. S7g). To sum up, these findings suggest that STAT4 directly represses the expression of PEBP1P2 by binding to the PEBP1P2 promoter region.

**PEBP1P2 stabilizes PEB1 mRNA**

To uncover the underlying mechanism of PEBP1P2-induced ccRCC metastasis promotion, we first analyzed the sequence of PEBP1P2 through the Encyclopedia of RNA Interactomes (ENCORI, [http://starbase.sysu.edu.cn/](http://starbase.sysu.edu.cn/)) [25]. In line with our conjecture, we found that PEBP1P2 might interact with PEB1 mRNA (Additional file 1: Fig. S8). PEB1 mediates ferroptosis and serves as a tumor-suppressor gene in various cancers. Knocking down PEBP1P2 decreased PEB1 mRNA and protein levels, and vice versa (Fig. 3a and b, Additional file 1: Figs. S9 and S10). Similarly, modulating STAT4 levels affected the mRNA and protein levels of PEBP1 (Additional file 1: Fig. S11). Surprisingly, PEBP1P2 did not appear to affect...
PEBP1 transcription or translation levels (Additional file 1: Fig. S12).

To demonstrate that PEBP1P2 directly interacts with PEBP1 mRNA, we performed a RIP assay based on MS2 stem-loop (MS2-RIP) using the empty vector and antisense expression plasmid as a negative control. The PEBP1 mRNA was highly enriched in the PEBP1P2-GFP complex (Fig. 3c and d). Besides, the RNA pulldown assay with the PEBP1P2 probe confirmed these results (Fig. 3e). To investigate whether this direct interaction with PEBP1P2 affected PEBP1 mRNA expression, we cloned the full-length PEBP1 mRNA and a PEBP1 mRNA mutant with a deletion of the binding site of PEBP1P2 and inserted them into the 3’ untranslated region (UTR) luciferase coding region (CDS). Wild-type plasmids altered luciferase activity, indicating PEBP1P2 expression changes, whereas mutants showed no significant change (Fig. 3f and g).

Next, to explore whether PEBP1P2 enhanced the stability of PEBP1 mRNA, we quantified PEBP1 and GAPDH in 786-O and A-498 cells transfected with PEBP1P2-shRNA or overexpressing vector relative to 0 h after blocking new RNA transcription with α-amanitin. The data are presented as the mean ± SD, ***P < 0.001.

Fig. 3 PEBP1P2 participates in the stable maintenance of PEBP1 mRNA. a, b PEBP1 mRNA levels were determined after transfection with shRNAs and overexpression vector by real-time PCR. c Assay model for MS2-RIP. d Real-time PCR was performed on RNA derived from MS2-RIP samples. The result of real-time PCR was normalized based on IgG control. e PEBP1 mRNA enrichment in PEBP1P2 complex was assessed by RNA pulldown assay and real-time PCR, and the mRNA enrichment was quantified by NC probe. f, g Luciferase activity of PEBP1-full-length and the PEBP1-mutation with the deletion of the binding site to PEBP1P2 was measured after transfected with indicated lentivirus. h–k Real-time PCR was performed to determine the stability of PEBP1 mRNA and GAPDH mRNA in 786-O and A-498 cells transfected with PEBP1P2-shRNA or overexpressing vector relative to 0 h after blocking new RNA transcription with α-amanitin. The data are presented as the mean ± SD, ***P < 0.001.
induced by silencing PEBP1P2, and vice versa (Additional file 1: Fig. S13). Aside from this, the analysis of the clinical sample and TCGA database indicated that a high PEBP1 expression correlated with a low PEBP1P2 expression in tumor tissues (Additional file 1: Fig. S14). These results confirm that PEBP1P2 mediates ccRCC metastasis formation by stabilizing PEBP1 mRNA.

PEBP1P2 prevents PEBP1 mRNA decay via RNA modification

Next, to explore the molecular mechanism of PEBP1P2-induced PEBP1 mRNA decay inhibition, we identified proteins potentially binding PEBP1P2 and PEBP1 mRNA using ENCORI. The analysis yielded nine protein candidates (Fig. 4a). Then, we designed shRNAs of these genes and transfected them into cells. Among the candidates, shELAVL1 downregulated PEBP1 mRNA the most (Fig. 4b, Additional file 1: Fig. S15). The luciferase assay yielded results consistent with this observation (Fig. 4c). Similarly, shELAVL1 downregulated the PEBP1 protein levels (Additional file 1: Fig. S16) and ELAVL1 reduced the half-life of PEBP1 mRNA (Additional file 1: Fig. S17). Besides, silencing PEBP1P2 hampered the interaction between ELAVL1 and PEBP1 mRNA, as shown by the MS2-RIP, RNA pulldown, and RIP assay results (Fig. 4d and e, Additional file 1: Fig. S18).

As an RNA-binding protein, ELAVL1 mediates RNA processing, such as maturation and degradation, via direct binding [26] to unmodified or modified RNA [27, 28]. In the PEBP1 mRNA sequence, we found some potential Nmethyladenosine (m^6^A), 5-methylcytosine (m^5^C), and N^2^-acetylcytidine (ac^4^C) modification sites. Then, we confirmed the m^5^A and m^5^C modifications on PEBP1 mRNA by m^5^A-, m^5^C-, and ac^4^C-RIP assays (Additional file 1: Fig. S19a–c). Next, to identify with which modified PEBP1 mRNA interacts, we constructed luciferase plasmids, each containing a different truncated fragment of PEBP1 mRNA, and co-transfected them with shPEBP1P2 or shELAVL1 into cells. PEBP1P2 and ELAVL1 mediated luciferase activity via the CDS and 3’-UTR of PEBP1 mRNA, which contained a large fraction of the binding site (Fig. 4f, g). Subsequently, we deleted or mutated the potential RNA modification sites and found that the m^5^C-deletion (Δm^5^C) and C-T-mutation, but not m^5^A-deletion (Δm^5^A) or A-G-mutation, eliminated shELAVL1-induced luciferase activity reduction (Fig. 4h, Additional file 1: Fig. S19d). Besides, the Δm^5^C or binding-region-deletion (ABR) hindered the interaction between PEBP1 mRNA and ELAVL1 (Fig. 4i, Additional file 1: Fig. S19e), and the PEBP1 mRNA containing Δm^5^C or ΔABR had short half-lives (Fig. 4j, Additional file 1: Fig. S19f).

To confirm that the m^5^C/m^6^A modifications were critical to producing PEBP1 mRNA, we used a targeted RNA demethylation system. The use of catalytically inactivated Cas13b (dCas13b) in conjunction with m^5^C/m^6^A erasers allowed us to add m^5^C/m^6^A modifications at sites specified through Cas13 gRNA. Full-length TET1/2- or ALKBH5-fused dCas13b with decreased gRNA targeted PEBP1 mRNA (gPEBP1) and enriched PEBP1 mRNA in m^5^C or m^6^A modifications (Additional file 1: Fig. S19g and h). However, only dCas13b-TET1/2 with gPEBP1 downregulated PEBP1 mRNA and impaired the interaction between PEBP1 mRNA and ELAVL1 (Fig. 4l, Additional file 1: Fig. S19i–l). Besides, dCas13b-TET1/2 with gPEBP1 promoted the decay of PEBP1 mRNA (Fig. 4m, Additional file 1: Fig. S19m and n).

In general, ELAVL1 binds to m^5^C by interacting with YBX1. The co-immunoprecipitation assay confirmed the interaction between ELAVL1 and YBX1 (Fig. 4n, o), and the absence of YBX1 decreased PEBP1 RNA and protein levels and reduced PEBP1 mRNA half-life (Fig. 4p, Additional file 1: Figs. S20, S21). Consistently, knocking down YBX1 or using YBX1 with a loss-of-function mutation weakened the interaction between ELAVL1 and PEBP1 mRNA (Fig. 4q and r). Aside from this, PEBP1 mRNA containing Δm^5^C/ΔABR inhibited the binding with YBX1 (Fig. 4s), just like transfection with dCas13b-TET1/2 and gPEBP1 (Additional file 1: Fig. S22). Meanwhile, ELAVL1

(See figure on next page.)

**Fig. 4** PEBP1P2 inhibits the decay of PEBP1 mRNA via RNA modification. a Venn diagram of the identified gene candidates which could interact with PEBP1 mRNA and PEBP1P2 predicted by ENCORI database. b The mRNA level of PEBP1 after transfection with indicated shRNAs was detected by real-time PCR, respectively. c Luciferase activity of PEBP1-full-length was measured after transfected with indicated lentivirus. d The RNA pulldown assay, MS2-RIP, and western blot were performed to determine whether protein candidates could bind PEBP1 RNA. e PEBP1 mRNA enrichment in the complex of protein candidates was assessed by RIP assay and real-time PCR, and the mRNA enrichment was quantified by IgG control. f–h Luciferase activity of PEBP1-full-length and the PEBP1-mutation with the depletion of indicated regions was measured after being treated with indicated lentivirus. i The ELAVL1 binding capacity to PEBP1 mRNA with indicated mutations was validated by MS2-RIP, RNA pulldown assay, and western blot. j Real-time PCR was used to determine the stability of PEBP1 and PEBP1P2 with indicated mutations and GAPDH transcripts in 786-O cells transfected with PEBP1P2-shRNA. k–l Following transfection with indicated lentiviruses, PEBP1 mRNA levels were determined by real-time PCR. m Real-time PCR was used to assess the stability of PEBP1 mRNA in 786-O cells transfected with indicated lentiviruses. n ELAVL1 and YBX1 were tested for interaction using the CoIP assay. o The interaction between YBX1 and PEBP1 mRNA was elevated using MS2-RIP, RNA pulldown assay, and western blot. p The mRNA levels of YBX1 and PEBP1P2 after transfection with shYBX1 were measured by real-time PCR. q–s After transfection with indicated lentiviruses, MS2-RIP, RNA pulldown assay, and western blot was performed to determine whether YBX1 and PEBP1 transcripts interact. The data are presented as the mean ± SD, **p < 0.001, n.s. no significance.
Fig. 4 (See legend on previous page.)
and YBX1 mRNA and protein levels did not significantly change (Additional file 1: Fig. S23). Overall, PEBP1P2 inhibited the decay of PEBP1 mRNA via m^5^C/YBX1/ELAVL1.

**PEBP1P2 mediates KLF13 by post-transcriptional regulation**

To find out whether PEBP1P2 could function as a miRNA sponge, we analyzed the PEBP1P2 sequence with ENCORI and identified 14 miRNAs. After alignment with TCGA data, we found that miR-296, miR-616, and miR-3194 were upregulated in ccRCC and correlated with poor prognosis (Additional file 1: Fig. S24). Silencing PEBP1P2 increased miR-296, miR-616, and miR-3194 levels, and overexpressing PEBP1P2 reduced them (Fig. 5a and b, Additional file 1: Fig. S25a and b). Conversely, upregulating miR-296, miR-616, and miR-3194 decreased the PEBP1P2 RNA levels, and inhibiting the miRNAs expression reduced them (Fig. 5c–f, Additional file 1: Fig. S25c, d). To confirm that their interconnected regulation depended on their direct interaction, we conducted AGO2-RIP, MS2-RIP, and RNA pulldown assays. miR-296, miR-616, and miR-3194 did directly bind to PEBP1P2 (Fig. 5g–k, Additional file 1: Fig. S25d). A luciferase assay confirmed this conclusion (Fig. 5l and m). Besides, PEBP1P2 appeared to play a vital role in the migration and invasion of cells by regulating miR-296, miR-616, and miR-3194 in the transwell assay (Additional file 1: Fig. S25f).

To identify the target mRNA of miR-296, miR-616, and miR-3194, we performed a conjoint ENCORI and miRWalk (http://mirwalk.umm.uni-heidelberg.de/) [29] analysis. We identified 69 mRNA candidates (Fig. 5n). Subsequently, we cloned the 3’-UTR, and inserted them into the pGLO-miR plasmid. Silencing PEBP1P2 yielded the most notable KLF13 activity reduction (Additional file 1: Fig. S26a). Besides, upregulating miR-296, miR-616, and miR-3194 lowered the KLF13 mRNA levels, while transfection with anti-miR-296, anti-miR-616, and anti-miR-3194 raised them (Fig. 5o, Additional file 1: Fig. S26b and c). In addition, PEBP1P2 regulated the KLF13 mRNA and protein levels along with miR-296, miR-616, and miR-3194 (Fig. 5p, Additional file 1: Fig. S26b–e). Next, we confirmed the direct interaction between KLF13 mRNA and miR-296, miR-616, and miR-3194 through AGO2-RIP, MS2-RIP, and RNA pulldown assays (Fig. 5q–s, Additional file 1: Fig. S26f). The luciferase assay further confirmed this result (Additional file 1: Fig. S26g). Besides, the transwell assay results indicated that miR-296, miR-616, and miR-3194 mediated the migration and invasion of cells by regulating KLF13 expression (Additional file 1: Fig. S27).

Correspondingly, the clinical sample analysis indicated that KLF13 expression was correlated with PEBP1P2 expression in tumor tissues (Additional file 1: Fig. S28). Overall, these data indicate that PEBP1P2 mediates KLF13 expression by acting as a sponge for miR-296, miR-616, and miR-3194. According to TCGA, PEBP1P2, PEBP1, and STAT4 were statistically associated with the prognosis of ccRCC patients, and PEBP1P2 could serve as a diagnosis and prognosis biomarker of ccRCC (Additional file 1: Fig. S29).

**Discussion**

For ccRCC with rich micro-vessels and lymphatic network, metastasis formation is frequent and surgical excision is currently the primary treatment option for patients with early-stage ccRCC [5]. Unfortunately, ccRCC patients are usually diagnosed at late stages. Hence, understanding the underlying mechanisms of ccRCC metastasis formation is urgent. The present study demonstrated that, in ccRCC, the high transcription factor STAT4 levels suppress the expression of PEBP1P2, promoting metastasis formation. Mechanistically, PEBP1P2 directly binds to PEBP1 mRNA and recruits YBX1 and ELAVL1 to PEBP1 mRNA, enhancing the stability of PEBP1 mRNA. PEBP1P2 also acts as a sponge for miR-296, miR-616, and miR-3194, preventing them from reducing KLF13 expression by directly interacting with KLF13 mRNA. Therefore, this study documents a novel
Fig. 5 (See legend on previous page.)
ccRCC regulatory mechanism involving \textit{PEBP1P2} and provides a new potential therapeutic strategy to overcome ccRCC metastasis formation (Fig. 6). Due to the small size of the validation cohort, our conclusions on the link between \textit{PEBP1P2} downregulation and ccRCC patients prognosis remains limited. Thus, we aim to follow up with a prospective cohort study to detail this relationship. This future study will help develop ccRCC diagnosis and treatment tools.

Pseudogenes, once seen as leftover information from evolution, are actually involved in tumor occurrence and development [11, 14, 15]. Open reading frame disruptions lead the transcriptional products of pseudogenes, without translation products, to function like long non-coding RNA [30]. It is well known that pseudogenes contribute to forming a competing endogenous RNA network. The \textit{UBE2CP3} pseudogene drives gastric cancer metastasis by sponging miR-138-5p and mediating ITGA2 expression [31]. Pseudogene \textit{CTSLP8}, acting as a sponge for miR-199a-5p, promotes ovarian cancer metastasis formation [32].

Interestingly, the \textit{WTAPP1} pseudogene promotes the translation of WTAP via direct binding with WTAP mRNA, facilitating pancreatic cancer progression [33]. Besides, pseudogenes can interact with proteins and regulate their expression or activation. \textit{PRELID1P6} mediates ubiquitin-mediated degradation of hnRNPH1 and promoting glioma proliferation [34]. Similarly, \textit{CMAHP} promotes gastric cancer metastasis formation by reducing the ubiquitination of Snail [35]. Our results show that \textit{PEBP1P2} stabilizes \textit{PEBP1} mRNA via direct binding and acts as a sponge for miR-296, miR-616, and miR-3194, preventing ccRCC metastasis formation.

RNA modification is an essential post-transcriptional mechanism that rapidly mediates RNA and protein levels without affecting DNA or histones. The regulation and function of the RNA methylation m^6A in human malignancies have been studied most intensively, revealing that m^6A affects various tumor processes [36, 37], such as proliferation [20, 21], stemness [38], and metastasis formation [39]. Besides, m^5C mediates mRNA stability and translation, exerting powerful effects in multiple tumor types [40]. Despite the fact that NSUN family members can serve as m^5C writers and TET1/2 participates in DNA/RNA demethylation [28, 41], very little information is available about m^5C readers and erasers. By stabilizing the mRNA of \textit{GRB2}, NSUN2 facilitates the development of esophageal squamous cell carcinoma [40]. The ALYREF, an m^5C-binding protein, stabilizes \textit{PKM2} mRNA, promoting bladder cancer tumorigenesis through PKM2-mediated glycolysis [42]. Here, we demonstrated that binding with \textit{PEBP1P2} elongated the
half-life of m\textsuperscript{5}C-modified PEBP1 mRNA. We revealed that the YBX1 and ELAVL1 complex stabilized PEBP1 mRNA, probably after recruitment by PEBP1P2.

Most RNA modification studies knock down or overexpress proteins of interest to assess the function of m\textsuperscript{6}A/m\textsuperscript{5}C. However, the global effect on all potential modification sites might affect the results. Li [43] and Wilson [44] designed and established a targeted m\textsuperscript{6}A RNA methylation system allowing to assess the effect of m\textsuperscript{6}A modifications at specific sites without affecting the levels of the protein of interest [20, 21]. Correspondingly, we designed and used a targeted m\textsuperscript{5}C RNA demethylation system to illustrate the effect of m\textsuperscript{5}C at specific PEBP1 mRNA sites.

The RNA-binding protein ELAVL1 has multiple functions via multiple binding modes. ELAVL1 directly binds to mRNA and affects its stability or alternative splicing via AU-rich elements [45, 46]. ELAVL1 could potentially function as a reader of m\textsuperscript{5}A to mediate the stability of RNA. ELAVL1 stabilizes ZMYM1 mRNA in an m\textsuperscript{6}A-dependent manner, enhancing gastric cancer progression by facilitating the transition from epithelial to mesenchymal tissue [47]. METTL3 stabilizes ARHGDA mRNA by modulating ELAVL1 expression in prostate cancer [27]. Here, we confirmed that ELAVL1 binds to RNA through m\textsuperscript{5}C, and YBX1 serves as a bridge. YBX1 binds to HDGF mRNA in an m\textsuperscript{5}C-depend manner, and ELAVL1 mediates the half-life of HDGF mRNA by interacting with YBX1 [28], promoting bladder cancer pathogenesis. Here, we showed that ELAVL1 and YBX1 increased the half-life of PEBP1 mRNA, inhibiting ccRCC progression.

Conclusions

In conclusion, our results demonstrate that the pseudogene PEBP1P2 significantly impacts ccRCC metastasis formation. A high STAT4 expression lowers PEBP1P2 expression, preventing PEBP1P2 from protecting PEBP1 mRNA by binding to it directly, recruiting the YBX1/ELAVL1 complex, suppressing the expression of KLF13 through sponging miR-296, miR-616, and miR-3194. These findings improve the understanding of the biological function and underlying mechanism of pseudogene PEBP1P2 in ccRCC metastasis formation and provide insights for RNA-based diagnosis and therapy of advanced ccRCC.

Supplementary Information

The online version contains supplementary material available at https://doi.org/10.1186/s40164-022-00346-2.

Additional file 1: Fig. S1. Low expression of pseudogene PEBP1P2 is linked to the advanced stage of ccRCC. a-d The RNA level of PEBP1P2 was analyzed according to different group in the ccRCC dataset TCGA database. The data are presented as the mean ± SD, *P< 0.05, **P< 0.01, ***P< 0.001. Fig. S2. Low expression of PEBP1P2 showed no effect on the progression of papillary RCC and chromophobe RCC. a-c Kaplan–Meier curve was conducted to estimate overall survival (a), disease specific survival (b) and progress free interval (c) in papillary RCC dataset of TCGA database. d-f Kaplan–Meier curve was conducted to estimate overall survival (d), disease specific survival (e) and progress free interval (f) in chromophobe RCC dataset of TCGA database. Fig. S3. The RNA level of PEBP1P2 after transfection with indicated lentivirus or ASOs a The RNA level of PEBP1P2 after transfection with indicated shRNAs. b The RNA level of PEBP1P2 after transfection with indicated overexpressing vector. c The RNA level of PEBP1P2 after transfection with indicated lentivirus or ASOs. d The RNA level of PEBP1P2 after transfection with indicated lentivirus or ASOs. e The protein level of PEBP1P2 after transfection with indicated lentivirus. The data are presented as the mean ± SD, ***P< 0.001. Fig. S4. PEBP1P2 reduces cell migration and invasion. a-c Migration and invasion assays were conducted with transfected cells using Transwell inserts. Fig. S5. Low expression of PEBP1P2 participates the formation of high migratory capacity. a Ten rounds of Transwell selection were conducted to screened out the 786-O cell with high migratory capacity (786-OhiMi) and low migratory capacity (786-OloMi), and migration and invasion assays were conducted to confirm the construction of these two cell lines. b The RNA level of PEBP1P2 after ten rounds of Transwell selection was detected by real-time PCR. c Migration and invasion assays were conducted with transfected cells using Transwell inserts. The data are presented as the mean ± SD, ***P< 0.001. Fig. S6. The mRNA level of 21 transcription factors were analyzed. The mRNA level of 21 transcription factors which could bind to the promoter region of PEBP1P2 were analyzed according to the all samples and the paired samples in the ccRCC dataset TCGA database. The data are presented as the mean ± SD, *P< 0.05, **P< 0.01, ***P< 0.001, n.s. = no significance. Fig. S7. The expression of PEBP1P2 is inhibited by STAT4. a The mRNA level of indicated genes after transfection with indicated shRNAs was detected by real-time PCR respectively. b The RNA level of PEBP1P2 after transfection with indicated shRNAs was detected by real-time PCR c The mRNA level of indicated genes after transfection with indicated shRNAs was detected by real-time PCR respectively. d The protein levels of STAT4 and FOXP3 were determined by western blot after overexpression of STAT4 and FOXP3. e-f The correlation between STAT4 and PEBP1P2 was analyzed according to the ccRCC dataset (e) and the clinical ccRCC sample (f). g Migration and invasion assays were conducted with transfected cells using Transwell inserts. The data are presented as the mean ± SD, ***P< 0.001. Fig. S8. The expression of potential mRNA binding with PEBP1P2 in ccRCC. a Heat map comparing the mRNA level of potential mRNA binding with PEBP1P2 in ccRCC and adjacent non-cancerous tissues. b-i Analysis of potential gene in ccRCC and adjacent non-cancerous tissues were performed using TCGA data and CPTAC data. The mRNA levels of 21 transcription factors were analyzed. The mRNA level of 21 transcription factors which could bind to the promoter region of PEBP1P2 were analyzed according to the ccRCC dataset TCGA database. The data are presented as the mean ± SD, *P< 0.05, **P< 0.01, ***P< 0.001. Fig. S9. The mRNA level of PEBP1P2 after transfection with indicated lentivirus or ASOs. a The mRNA level of PEBP1P2 after transfection with indicated shRNAs was detected by real-time PCR. b The RNA level of PEBP1P2 after transfection with indicated lentivirus or ASOs. c The protein level of PEBP1P2 was detected by western blot after overexpression of STAT4 and FOXP3. d-f The mRNA and protein level of PEBP1P2 were determined by western blot after overexpression of indicated shRNAs. e The mRNA level of PEBP1P2 was determined by western blot after overexpression of indicated shRNAs. f The protein level of PEBP1P2 was determined by western blot after overexpression of indicated shRNAs. The data are presented as the mean ± SD, ***P< 0.001. Fig. S10. The protein level of PEBP1P2 after transfection with indicated lentivirus or ASOs. a The mRNA level of PEBP1P2 after transfection with indicated shRNAs was detected by real-time PCR. b The mRNA level of PEBP1P2 after transfection with indicated shRNAs was detected by real-time PCR. c The mRNA level of PEBP1P2 was determined by western blot after overexpression of indicated shRNAs. The data are presented as the mean ± SD, ***P< 0.001. Fig. S11. The protein level of PEBP1P2 after transfection with indicated lentivirus. The data are presented as the mean ± SD, ***P< 0.001. Fig. S12. The protein level of PEBP1P2 after transfection with indicated shRNAs was detected by real-time PCR. The data are presented as the mean ± SD. a The protein level of PEBP1P2 after transfection with indicated lentivirus or ASOs. b The RNA level of PEBP1P2 after transfection with indicated shRNAs was detected by real-time PCR. c The mRNA level of PEBP1P2 was determined by western blot after overexpression of indicated shRNAs. The data are presented as the mean ± SD. a The protein level of PEBP1P2 after transfection with indicated lentivirus or ASOs. b The RNA level of PEBP1P2 after transfection with indicated shRNAs was detected by real-time PCR. c The mRNA level of PEBP1P2 was determined by western blot after overexpression of indicated shRNAs. The data are presented as the mean ± SD. a The RNA level of PEBP1P2 after transfection with indicated lentivirus or ASOs. b The RNA level of PEBP1P2 after transfection with indicated shRNAs was detected by real-time PCR. c The mRNA level of PEBP1P2 was determined by western blot after overexpression of indicated shRNAs. The data are presented as the mean ± SD. a The RNA level of PEBP1P2 after transfection with indicated lentivirus or ASOs. b The RNA level of PEBP1P2 after transfection with indicated shRNAs was detected by real-time PCR. c The mRNA level of PEBP1P2 was determined by western blot after overexpression of indicated shRNAs. The data are presented as the mean ± SD. a The protein level of PEBP1P2 after transfection with indicated lentivirus. The data are presented as the mean ± SD, ***P< 0.001. Fig. S13. PEBP1P2 reduces cell migration and invasion via regulating the expression of PEBP1. Migration and invasion assays were conducted with transfected indicated lentivirus, via Transwell inserts. Fig. S14. PEBP1P2 is low expressed in ccRCC. The mRNA level of PEBP1P2 in 21 pairs of human clinical ccRCC tissues normalized to corresponding adjacent.
non-cancerous tissues were detected by real-time PCR. The correlation between PEBP1P2 and PEBP1 was analyzed using the clinical dataset TCGA database. The datasets used and/or analyzed during the current study are available from the corresponding author upon reasonable request.

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Author contributions
LY, HLY and YC conducted all experiments and analyzed the data. DMH, HQG and WDG designed the whole project and supervised all experiments. CP, HXH, YWL, WLM and XL provided support with experimental and clinical techniques. All authors read and approved the final manuscript.

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Availability of data and materials
The datasets used and/or analyzed during the current study are available from the corresponding author upon reasonable request.

Declarations
Ethical Approval and Consent to participate
The present study was approved by the Medical Ethics Committee of Affiliated Drum Tower Hospital of Medical School of Nanjing University.

Consent for publication
Not applicable.
Competing interests
The authors declare that they have no competing interests.

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