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Original Article

CDK1 promotes the phosphorylation of KIFC1 to regulate the tumorgenicity of endometrial carcinoma

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ABSTRACT

Objective: This study aims to clarify the mechanical action of cyclin-dependent protein kinase 1 (CDK1) in the development of endometrial carcinoma (EMCA), which may be associated with the phosphorylation of kinesin family member C1 (KIFC1) and further activate the PI3K/AKT pathway.
Methods: The protein and gene expression of CDK1 in EMCA tissues and tumor cell lines were evaluated by western blot, quantitative polymerase chain reaction, and immunohistochemistry staining. Next, Cell Counting Kit-8 and colony formation assay detected cell survival and proliferation. Cell migration and invasion were measured by Transwell assay. Cell apoptosis and cell cycle were tested by flow cytometry. Immunofluorescence staining of γH2AX was used to evaluate DNA damage, respectively. Subsequently, a co-immunoprecipitation assay was used to detect the interaction between CDK1 and KIFC1. The phosphorylated protein of KIFC1 and PI3K/AKT was detected by western blot. Finally, the effect of CDK1 on the tumor formation of EMCA was evaluated in a nude mouse xenograft model.
Results: CDK1 was highly expressed in EMCA tumor cell lines and tissues, which contributed to cell survival, proliferation, invasion, and migration, inhibited cell apoptosis, and induced DNA damage of EMCA cells dependent on the phosphorylation of KIFC1. Moreover, the CDK1-KIFC1 axis further activated PI3K/AKT pathway. Finally, CDK1 knockdown repressed tumor formation of EMCA in vivo.
Conclusion: We report that increased CDK1 promotes tumor progression and identified it as a potential prognostic marker and therapeutic target of EMCA.

Keywords: Cyclin-Dependent Protein Kinase 1; KIFC1; PI3K/AKT; Tumor Progression; Endometrial Carcinoma

Synopsis

The promoting effect of cyclin-dependent protein kinase 1 (CDK1) on endometrial carcinoma progression by targeting the kinesin family member C-mediated PI3K/AKT signaling pathway to increase cell growth, metastasis, and DNA damage and restrict cell apoptosis and cell cycle, which indicated the oncogenic ability of CDK1.

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Conflict of Interest
No potential conflict of interest relevant to this article was reported.

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INTRODUCTION

Endometrial carcinoma (EMCA) is one type of gynecological cancer originating from endometrial epithelial tissues [1]. Due to its high proliferation and invasion ability, it has become one of the most diagnosed and prevalent gynecological malignancies worldwide [2,3]. The average lifetime risk of EMCA is almost 3%, with a median age at diagnosis of 61 years [4]. Furthermore, the mobility and mortality of EMCA are increasing globally [5]. Despite the rapid development of technology and advanced prognosis, the etiology and pathogenesis of EMCA remain poorly defined. Therefore, exploring the pathogenesis of EMCA and finding effective therapeutic targets are essential for improving the survival rate of EMCA patients.

Cancers originate from uncontrolled cell proliferation and disturbed cell cycles, regulated by abnormal activation of a series of proteins [6]. CDK1, encoded by cell division cycle gene 2, belongs to the cyclin-dependent protein kinases (CDKs) family and participates in the process of the cell cycle [2]. A previous study has concluded that CDK1 contributed to S/G2 and G2/M transitions and G2 progression to regulate the cell cycle process [7,8]. Moreover, CDK1 participated in cell proliferation and cellular apoptosis in various cancers, including breast cancer [9] and hepatocellular carcinoma [8]. Due to the important role of CDKs on cell viability, proliferation, and cell cycle, CDKs have been regarded as the natural targets for anticancer therapy [7]. However, the effect of CDK1 on the tumorigenicity of EMCA is vague and must be fully illustrated, and the potential mechanism needs further investigation.

The kinesin family member C1 (KIFC1), belonging to the kinesin-14 family, plays a pivotal role in various physiological processes, such as mitosis [10], oocyte development [11], spermatogenesis [12], and DNA transport [13]. Recently, highly expressed KIFC1 was found in different cancer cells and tumor tissues, and KIFC1 participated in cell division and proliferation to promote tumor development [6,10,14]. Our previous study also showed that KIFC1 contributed to cell proliferation and invasion [1] and affected cell metabolism in EMCA models [15]. Thus, the inhibition of KIFC1 may be a potential target in cancer therapy. However, whether KIFC1 takes part in the process of CDK1 contributing to the development of EMCA needs further investigation.

In our project, we first detected the protein and mRNA expression of CDK1 in EMCA cells and human tissues. In addition, the regulatory effect of CDK1 on cell survival, proliferation, invasion, migration, and apoptosis was evaluated in vitro, as well as the potential mechanism. The results based on the above experiments inferred that the expression of CDK1 was higher in EMCA cells and human patients' tissues. Moreover, CDK1 promoted cell growth, proliferation, invasion, migration, and cell cycle arrest and inhibited cell apoptosis dependent on KIFC1 phosphorylation. Mechanically, CDK1-mediated KIFC1 phosphorylation activated PI3K/AKT pathway, thereby promoting the development and progression of EMCA. The anticancer property of CDK1 knockdown was proved in xenograft mice to inhibit tumor growth and cell proliferation, making it a potential target for treatment for EMCA.

MATERIALS AND METHODS

1. Patients and tissue samples

EMCA tissues and matched adjacent normal tissues were collected from 10 patients at The Quzhou Affiliated Hospital of Wenzhou Medical University, Quzhou People's Hospital. The

detailed characteristics of patients are shown in **Table S1**. These patients did not receive chemotherapy or radiotherapy before the operation. Prior informed consent from the patients and ethics approval from the Medical Ethics Review Committee of Quzhou People's Hospital (approval No. 2022-113) for this research.

2. Cell culture

The following human endometrial cancer cell lines, including Ishikawa, HEC-1B, HEC-1A, and KLE cells, normal human endometrial endothelial cell line (HeEC), and HEK 293T were purchased from American Type Culture Collection. The Ishikawa cells were maintained in modified Eagle's medium (MEM; Gibco, Waltham, MA USA) supplemented with 5% fetal bovine serum (FBS; Sigma-Aldrich, St. Louis, MO, USA) and 2% penicillin/streptomycin (100 U/mL; Biological Industries, Kibbutz Beit-Haemek, Israel). The HEC-1B, HEC-1A, and HEK 293T cells were cultured in Dulbecco's Modified Eagle Medium (DMEM; Gibco) basic supplemented with 10% FBS and 1% penicillin/streptomycin. The KLE cells were cultured in DMEM/F12 (Gibco) supplemented with 10% FBS and 2% penicillin/streptomycin. In addition, HeEC cells were inoculated in Roswell Park Memorial Institute-1640 (Invitrogen, Waltham, MA, USA) with 10% FBS and 1% penicillin/streptomycin. All cell lines were tested twice a month for mycoplasma by the LookOut Mycoplasma PCR Detection Kit (Sigma-Aldrich) and incubated at 37°C in a humidified atmosphere of 5% CO_2.

3. Plasmid construction, lentiviral short-hairpin RNAs, and cell transfection

Full-length cDNA encoding human CDK1 and KIFC1 were polymerase chain reaction (PCR)-amplified and cloned into pcDNA3.1 vector (GenScript, Piscataway, NJ, USA) to upregulate their expressions and then were transfected into Ishikawa and HEC-1A cells for exosmotic study. PcDNA 3.1 vector (Vector), pcDNA3.1-CDK1 (oeCDK1), and pcDNA3.1-KIFC1 (oeKIFC1) originated from GenScript (Shanghai, China) according to their instructions [16]. Two short hairpin RNA (shRNA) oligonucleotide sequences targeting human CDK1 were synthesized by GenScript and transfected into Ishikawa and HEC-1B cells to silence endogenous CDK1. Nonsense shRNA was used as a negative control (shNC). For the co-immunoprecipitation (Co-IP) assay, the total length of CDK1 was cloned into Flag-FBXW7 plasmid (GenScript), and the entire length of KIFC1 was cloned into Myc-MLST8 (GenScript) according to their instructions.

EMCA cells were cultured to 50% confluence in 6 cm dishes and transfected with different vectors using Lipofectamine 3000 (Invitrogen) to overexpress or knock down the expression of related genes. As in the previous study, the cells were harvested after 24 hours for transfection and used for later analysis [16]. For the animal study, cell infection with shNC or shCDK1 was performed using the lentivirus in Ishikawa cells; stable-expressing cells were then sorted out by puromycin and used for nude mice.

4. Quantitative PCR (qPCR) measurement

Total RNA, extracted from human tissue samples (normal and tumor tissues) and cultured cells for qPCR, was isolated using TRIzol reagent (Invitrogen) according to the manufacturer's protocol [17]. Total RNA was reverse transcribed into first strand cDNA using RevertAid RT Reverse Transcription Kit (Thermo Fisher Scientific, Waltham, MA, USA). qPCR was conducted with FastStart Universal SYBR Green master mix (Roche, Mannheim, Germany) via StepOnePlus Real-Time PCR system (Applied Biosystems, Foster City, CA, USA). Each sample was run in triplicate and normalized to the reference glyceraldehyde 3-phosphate dehydrogenase (GAPDH) gene expression. The relative mRNA expression of

CDK1 was calculated using the $2^{-\Delta\Delta Ct}$ method. The gene-specific primers were designed via NCBI Primers BLAST and utilized to measure CDK1 expression:

CDK1-F: 5′- AAAATTGGAGAAGGTACCTATGGA -3′
CDK1-R: 5′- CCCTTCCTCTTCACTTTCTAGTCTG -3′
GAPDH-F: 5′- CTGGGCTACACTGAGCACC -3′
GAPDH-R: 5′- AAGTGGTCGTTGAGGGCAATG -3′

5. Western blot analysis

Human EMCA cells (Ishikawa, HEC-1B, HEC-1A, and KLE) and HeEC were collected and lysed in RIPA buffer (Beyotime Biotechnology, Shanghai, China) supplemented with a protease inhibitors cocktail (Roche, Basel, Switzerland). Protein concentrations were determined by a standard BCA protein assay kit (KeyGEN BioTECH, Nanjing, China). The procedures of this part were conducted according to previous studies as follows [17,18]. Equal proteins were subjected to sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis and electroblotted onto polyvinylidene fluoride membranes (Bio-Rad Laboratories, Hercules, CA, USA), blots were then blocked at room temperature in 5% nonfat dry milk solution (Beyotime Biotechnology) for 1 hour. Subsequently, the blots were probed with the following primary antibodies to CDK1 (1:5,000, No. 19532-1-AP; Proteintech, Rosemont, IL, USA), KIFC1 (1:1,000, No. 20790-1-AP; Proteintech), p-KIFC1 (phosphorylated antibody; Huabio, Woburn, MA, USA), γH2AX (1:2,000, ab26350; Abcam, Cambridge, UK), PI3K (1:1,000, ab191606; Abcam), p-PI3K (1:1,000, ab182651; Abcam), AKT (1:10,000, ab179463; Abcam), p-AKT (1:1,000, ab192623; Abcam), Myc (1:1,000, #2276; Cell Signaling Technology, Danvers, MA, USA), Flag (1:1,000, F3165; Sigma-Aldrich) and GAPDH (1:10,000, ET1601-4; Huabio) at 4°C overnight. After washing 3 times and inoculating with horseradish peroxidase-conjugated goat-anti-rabbit (1:50,000, HA1001; Huabio) or goat-anti-mouse secondary antibodies (1:50,000, HA1006; Huabio) for 1 hour at room temperature, protein bands were visualized using an ECL luminescence reagent (Tanon, Shanghai, China). The bands were then quantified by ImageJ software (National Institutes of Health, Bethesda, MD, USA).

6. Cell viability assay

Ishikawa and HEC-1A cells were cultured in 96-well plates at 2×10^4 cells/well overnight and transfected with different plasmids for 24 hours. Cell viability was detected by the Cell Counting Kit-8 (CCK-8) assay (Beyotime Biotechnology) following the standard protocol. Viable cells were calculated at OD_{450} using synery^{TM}H1 Muti-Mode Reader spectrophotometry (Thermo Fisher Scientific).

7. Colony formation assay

A previous study described the colony formation assay protocol [14]. Briefly, Ishikawa, HEC-1B, and HEC-1A cells, transfected with shRNA and overexpressing plasmids for 24 hours, were counted and seeded into 6 cm dishes. After 14 days, the cells were fixed with methanol and stained with 0.1% crystal violet (Sigma-Aldrich) in 20% methanol for 20 minutes. Visible colonies were then manually counted. The number of colonies formed for each group was photographed and measured in triplicate.

8. Invasion and migration assays

The cells (Ishikawa, HEC-1B, and HEC-1A) were transfected with different plasmids for 24 hours for the cell invasion assay. Treated cells (1×10^5 /well) were suspended in a serum-free medium and then seeded in the upper layer of a trans-well chamber (6-well), which was precoated with 50 μL Matrigel (Becton Dickinson, Franklin Lakes, NJ, USA). The cells were

allowed to invade for another 24 hours. A complete medium (600 μL) with 15% FBS was added to the lower chamber. The invaded cells on the lower surface of the membrane were then fixed with 4% paraformaldehyde (PFA) for 30 minutes and stained with 0.1% crystal violet solution for 15 minutes. Using a Zeiss Microscope associated with a Nikon camera, 6 fields were randomly selected to calculate the invading cell area. Aside from Matrigel not being applied to the upper layer of the chamber for the cell migration experiment, the other procedures were the same as the cell invasion assay.

9. Flow cytometric analysis of apoptosis and cell cycle

Cell apoptosis was analyzed by FITC Annexin V Apoptosis Detection Kit (BD Biosciences, San Diego, CA, USA). Ishikawa and HEC-1A cells (1×10^5 cells/well in a 6-well plate) were trypsinized without EDTA 72 hours after transfection with various plasmids, followed by washing 2 times with phosphate buffered saline (PBS), resuspending in the binding buffer, and incubation in staining buffers (Annexin V-FITC and PI) for 15 minutes. The proportion of cell apoptosis was analyzed with a flow cytometer (BD Biosciences) within 1 hour after staining. All procedures were performed according to the manufacturer's protocol [19]. The images were analyzed with FlowJo software (Ashland, OR, USA). The early and late apoptotic cell quantities are shown in the Q3 and Q2 quadrants.

For the cell cycle analysis, Ishikawa and HEC-1A cells were transfected with vectors for 24 hours. Following treatments, cells were collected and fixed in 70% ice-cold ethanol for 24 hours. Then, cells were incubated in a staining buffer (PBS containing 20 μg/mL PI and 10 μg/mL RNase A) for 30 minutes. Finally, the cell cycle was detected by BD FACSCalibur flow cytometer. The ratio of cells in the G0/G1, S, and G2/M phases was counted and further analyzed by FlowLogic (Miltenyi Biotec, Bergisch Gladbach, Germany) software.

10. Histology and immunohistochemistry (IHC) staining

The adjacent and tumor tissues of EMCA patients and lung tissues of xenograft mice were fixed with 4% PFA for 24 hours at room temperature. The samples were then sent to Servicebio (Wuhan, China) for staining hematoxylin and eosin (H&E).

The adjacent and tumor tissues of EMCA patients and the tumors of mice were harvested and fixed in 4% PFA for 24 hours at room temperature. The samples were then sent to Servicebio for IHC analysis. The antibodies anti-CDK1 (1:200, No. 19532-1-AP; Proteintech), anti-KIFC1 (1:200, No. 20790-1-AP; Proteintech) and anti-Ki-67 (1:500, #12202; Cell Signaling Technology) were used in this part. Images of stained specimens, including H&E and IHC, were captured using an Olympus BX51 microscope (Olympus, Tokyo, Japan).

11. Immunofluorescence (IF) staining

IF staining was performed as previously described [20]. Briefly, Ishikawa and HEC-1A cells were transfected with vectors for 24 hours, washed once in PBS, fixed with cold methanol, permeabilized, incubated with primary antibody phosphor-H2AX (γH2AX), and followed by a secondary antibody (Goat anti-mouse IgG H&L Alexa Fluor 488, 1: 600, ab150113; Abcam). The images were collected by a confocal microscope (Zeiss LSM710; Zeiss, Oberkochen, Germany).

12. Protein Co-IP

Flag-CDK1 and Myc-KIFC1 vectors were generated by GenScript. After transfection for 24 hours, cells were centrifuged and harvested and then resuspended in 2× volume of IP lysis buffer (Pierce) supplemented with protease inhibitors cocktail (Roche) on ice for 10 minutes. After

that, the samples were incubated with the primary antibodies anti-Flag and anti-Myc along with Protein A/G magnetic beads overnight at 4°C. The beads were washed, resuspended in an SDS sample buffer, and analyzed by western blot analysis [20].

13. Nude mice experiments

The effect of CDK1 on promoting EMCA tumor cell growth was accessed using an in vivo nude mice model. Six weeks-old female BALB/c nude mice were purchased from the Laboratory Animal Center of the Chinese Academy of Sciences (Shanghai, China) for study. Mice were maintained under specific pathogen-free conditions under a 12-hour light/dark cycle for 7 continuous days before use. The Experimental Animal Ethics Committee of Wenzhou Medical University authorized the study and followed the National Institutes of Health Guidelines on the Care and Use of Animals (approval number: xmsq2022-0913).

Ishikawa cells (1×10^7 cells/site) that stably expressed shNC or shCDK1 were resuspended in 200 μL PBS mixed with 200 μL Matrigel (Corning, 354248) and then were subcutaneously injected into the right flank of mice. The tumor volume was measured using an external caliper and calculated using the formula $(L \times W^2)/2$, where L is the tumor length, and W is the width. After the mice were sacrificed 3 weeks later, the isolated tumors were photographed, weighed, and subjected to further pathological analysis. IHC staining was used to access the contents of CDK1 and KIFC1, while the proliferation was quantified by calculating the Ki-67-positive cells.

14. Statistical analysis

At least 3 independent experiments were conducted to obtain effective data, and results were presented as the mean ± standard error of mean. For example, #2 and #3 in all figures mean the biological replicates in our study. GraphPad Prism version 9.0 (GraphPad Software Inc., San Diego, CA, USA) was used for statistical analysis and drawing. Statistical significance was assessed by a 2-tailed Student's t-test (comparison between 2 groups) or analysis of variance (comparisons among 3 groups or more). $p<0.05$ was regarded as significance, and $p<0.01$ was regarded as extremely significance statistically.

RESULTS

1. CDK1 is highly expressed in EMCA tumor cells and tissues of EMCA patients and promotes the growth and proliferation of EMCA cells

To explore whether CDK1 participated in the progression of EMCA, we first detected the relative expression of CDK1 in EMCA cell lines (Ishikawa, HEC-1B, HEC-1A, and KLE) and normal HeEC by qPCR and western blot (**Fig. 1A**). Results indicated that the gene and protein expressions of CDK1 were pronouncedly higher in all EMCA cells. H&E staining of adjacent and tumor tissues of EMCA patients indicated an obvious inflammatory injury and infiltration (**Fig. 1B**). Moreover, IHC staining further verified that the content of CDK1 was increased in patients' tumors with EMCA, while CDK1 staining was almost undetectable in adjacent normal tissues (**Fig. 1B**). The above results suggested that CDK1 was upregulated in EMCA cells and tissues.

To further elucidate the role of CDK1 in EMCA cells, shCDK1 plasmids were transfected into Ishikawa and HEC-1B cells (among 4 types of tumor cells, whose CDK1 showed the top level), and CDK1 overexpression vector was transfected into HEC-1A cells (among 4 types of tumor

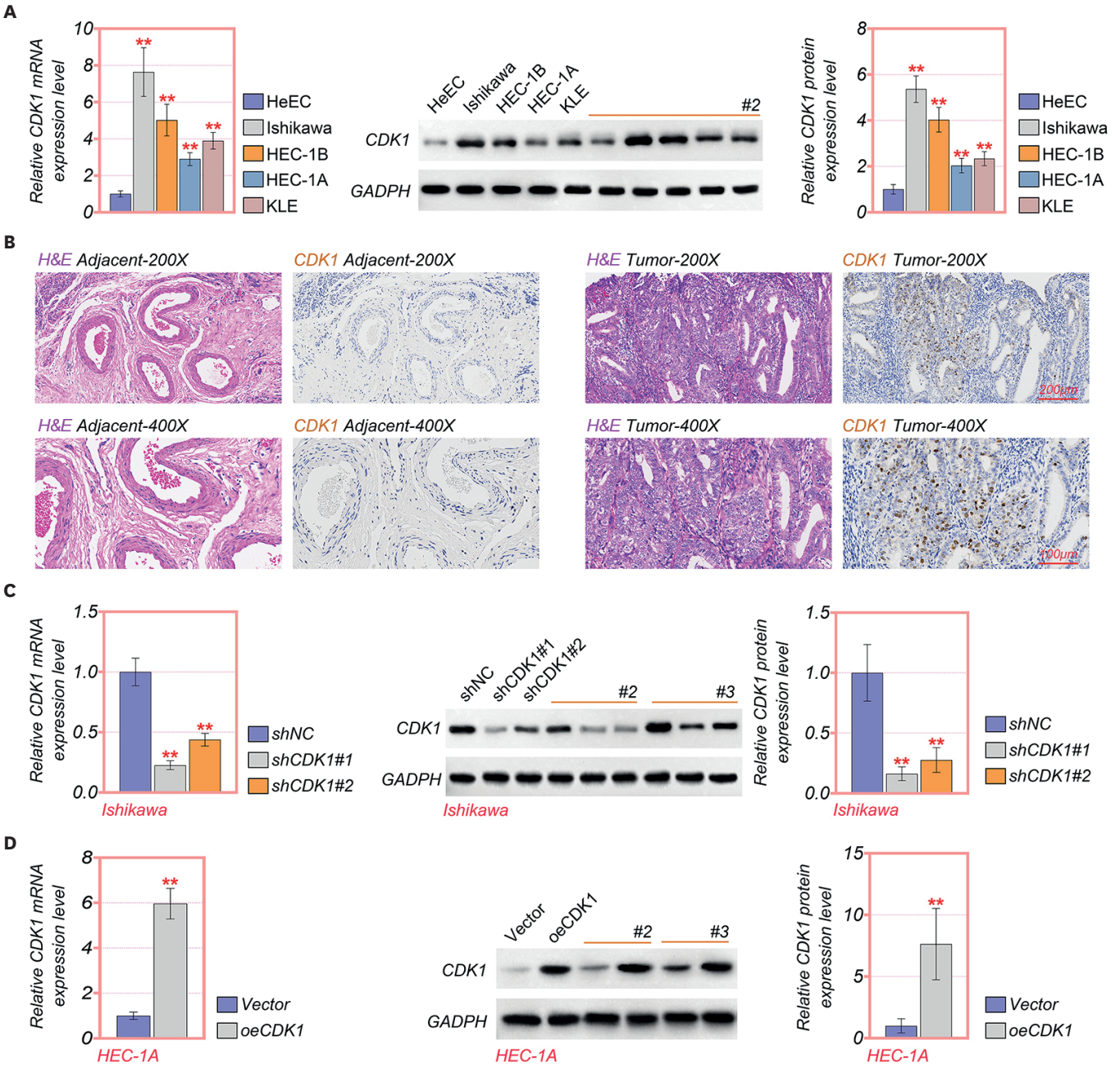

Fig. 1. CDK1 is highly expressed in EMCA tumor cells and tissues of EMCA patients. (A) qPCR and western blotting analysis of CDK1 expression in the normal cells and EMCA tumor cell lines. GAPDH was used as a housekeeping gene and loading control. (B) Representative H&E and immunohistochemistry images of CDK1 in paired normal tissues and EMCA tissues (scale bar: 200 μm for 200×; 100 μm for 400×). (C) The transfection efficiency of CDK1 knockdown in Ishikawa cells was evaluated by qPCR and western blotting analysis. (D) The transfection efficiency of CDK1 overexpression in HEC-1A cells was assessed by qPCR and western blotting analysis. #2 and #3 were repetitive experiments.
CDK1, cyclin-dependent protein kinase 1; EMCA, endometrial carcinoma; GAPDH, glyceraldehyde 3-phosphate dehydrogenase; HeEC, human endometrial endothelial cell line; H&E, hematoxylin and eosin; oeCDK1, pcDNA3.1-cyclin-dependent protein kinase 1; shCDK1, short hairpin cyclin-dependent protein kinase 1; shNC, short hairpin RNA negative control; qPCR, quantitative polymerase chain reaction.
The comparison between 2 groups: **p<0.01.

cells, whose CDK1 showed the lowest level) (**Fig. 1C and D**). Results showed that sh-CDK1#1 and sh-CDK1#2 plasmids significantly reduced the gene and protein expressions of CDK1 in Ishikawa cells, of which sh-CDK1#1 showed a more substantial effect (**Fig. 1C**). Thus, sh-CDK1#1 was used in the following experiments named shCDK1. HEC-1B cells transfected

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with shCDK1 vector also showed significant downregulation of CDK1 in mRNA level compared with the shNC vector (**Fig. S1B**). HEC-1A cells transfected with the CDK1 vector showed an apparent increase of CDK1 in the case of mRNA and protein levels (**Fig. 1D**).

The effect of CDK1 on EMCA cells was measured based on the above shCDK1 and oeCDK1 plasmids in different cell lines. Results in **Fig. 2A and B** showed that CDK1 knockdown pronouncedly inhibited cell viability and proliferation of Ishikawa cells. Similarly, CDK1 silencing also reduced cell colony formation of HEC-1B cells (**Fig. S1C**). CDK1 overexpression in HEC-1A cells consistently enhanced cell survival and promoted proliferation (**Fig. 2A and B**). Transwell assays indicated that CDK1 knockdown substantially restrained the migration and invasion of Ishikawa and HEC-1B cells (**Fig. 2C**, **Fig. S1D**), and CDK1 overexpression reversely drove the migration and invasion of HEC-1A cells (**Fig. 2D**). In addition, Ishikawa cells with shCDK1 vector increased the percentage of double positive cells representing apoptotic cells compared to Ishikawa cells with shNC vector (**Fig. 2E**). Similarly, HEC-1A cells with oeCDK1 vector significantly reduced the apoptotic cells compared to the Vector group (**Fig. 2E**).

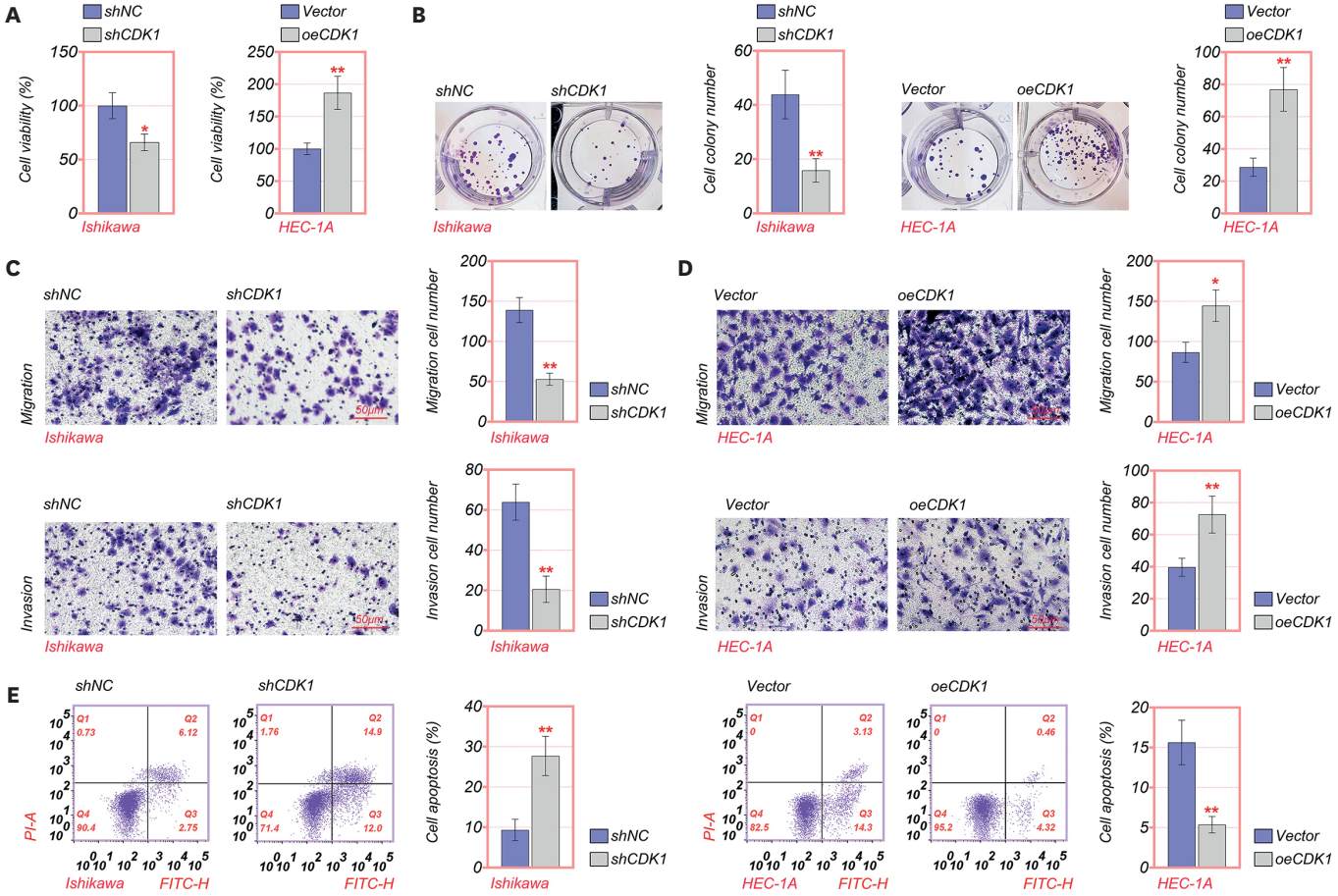

Fig. 2. CDK1 promotes the growth and proliferation of EMCA. (A) The cell viability of Ishikawa cells with CDK1 knockdown and HEC-1A cells with CDK1 overexpression was accessed by Cell Counting Kit-8 assay. (B) Representative images and quantification of colonies formed by Ishikawa cells with CDK1 knockdown and HEC-1A cells with CDK1 overexpression. (C) Representative images and quantification of cell migration and invasion by Transwell assays in Ishikawa cells with CDK1 knockdown (scale bar: 50 μm). (D) Representative images and quantification of cell migration and invasion by Transwell assays in HEC-1A cells with CDK1 overexpression (scale bar: 50 μm). (E) Detection of apoptotic cells by flow cytometric staining with Annexin V-FITC and PI in Ishikawa cells with CDK1 knockdown and HEC-1A cells with CDK1 overexpression.
CDK1, cyclin-dependent protein kinase 1; EMCA, endometrial carcinoma; oeCDK1, pcDNA3.1-cyclin-dependent protein kinase 1; shCDK1, short hairpin cyclin-dependent protein kinase 1; shNC, short hairpin RNA negative control.
The comparison between 2 groups: *p<0.05, **p<0.01.

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These results showed that CDK1 especially drove cell survival, proliferation, invasion, and migration and inhibited cell apoptosis, suggesting CDK1 could act as a promoting factor in EMCA cell growth in vitro.

2. CDK1 leads to DNA damage of EMCA cells in vitro

We then further explored the regulatory effect of CDK1 on cell cycle and DNA damage. γH2AX, a typical marker of DNA damage, was evaluated in our research. Our study showed that silencing of CDK1 in Ishikawa cells pronouncedly reduced the level of γH2AX by IF staining (**Fig. 3A**). In contrast, CDK1 overexpression in HEC-1A cells increased the level

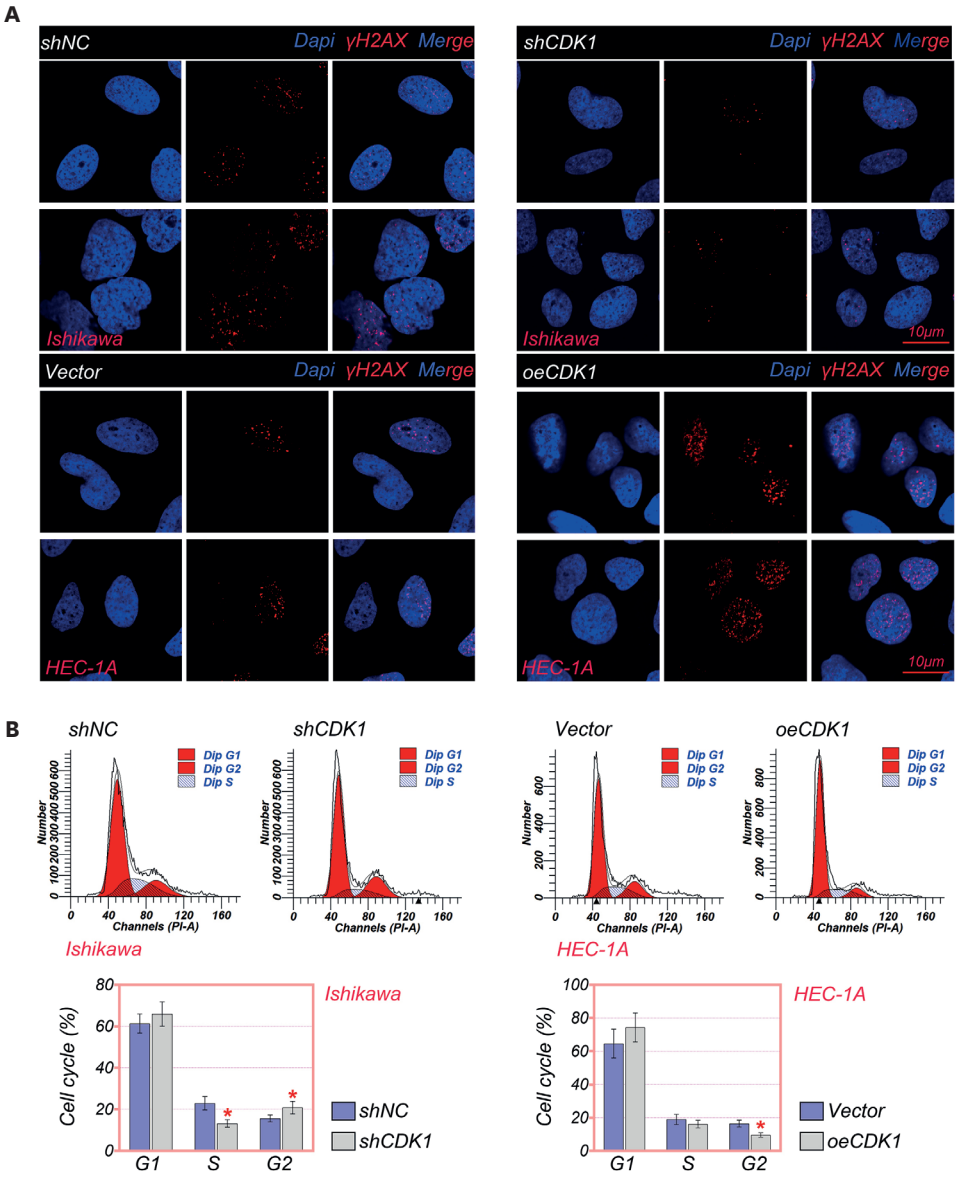

Fig. 3. CDK1 leads to DNA damage of EMCA cells in vitro. (A) Immunofluorescence staining of γH2AX in Ishikawa cells with CDK1 knockdown and HEC-1A cells with CDK1 overexpression (scale bar: 10 μm). (B) Analysis of different cell cycle stages and percentage of cell population in each stage in Ishikawa cells with CDK1 knockdown and HEC-1A cells with CDK1 overexpression by flow cytometry assay.
CDK1, cyclin-dependent protein kinase 1; EMCA, endometrial carcinoma; oeCDK1, pcDNA3.1-cyclin-dependent protein kinase 1; shCDK1, short hairpin cyclin-dependent protein kinase 1; shNC, short hairpin RNA negative control.
The comparison between 2 groups: *$p<0.05$.

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of γH2AX (**Fig. 3A**), indicating a promoting effect of CDK1 on DNA damage. Besides, the knockdown of CDK1 in Ishikawa cells was accompanied by a corresponding increase in the proportion of cells in the G2/M phase, a decrease in the proportion of cells in the S phase, and no noticeable change of G0/G1 phase (**Fig. 3B**). In contrast, overexpression of CDK1 in HEC-1A cells showed no impact on cells in the G0/G1 and S phases but significantly reduced the proportion of cells in the G2/M phase (**Fig. 3B**). These findings demonstrated that CDK1 led to DNA damage and cell cycle arrest in the G2/M phase to promote cancer development.

3. CDK1 regulates the growth and proliferation of EMCA cells dependent on KIFC1 in vitro

Since KIFC1 participated in the development of several cancers and correlated with poor prognosis of patients [6,10,14], our previous study reported that KIFC1 accelerated the proliferation and invasion of EMCA cells [1]. Thus, we further explored whether KIFC1 was involved in the process of CDK1 in regulating cell growth and proliferation in vitro. Clinically, the contents of KIFC1 in tumors were significantly higher than that in adjacent tissues of EMCA patients as accessed by IHC staining (**Fig. S1A**), while H&E staining also showed marked pathological injury (**Fig. S1A**), similar to the results of **Fig. 1B**. Moreover, our results also showed that silencing of CDK1 in Ishikawa cells markedly reduced the protein level of p-KIFC1/KIFC1 (**Fig. 4A**), whereas CDK1 overexpression in HEC-1A cells markedly increased the level of p-KIFC1/KIFC1 (**Fig. 4A**), suggesting that silencing of CDK1 in Ishikawa cells substantially inhibited the phosphorylation of KIFC1, and CDK1 overexpression reversely drove the protein level of KIFC1 phosphorylation in HEC-1A cells (**Fig. 5B**), which again demonstrated that KIFC1 phosphorylation showed an essential effect in the process of CDK1 regulating tumor cells. Besides, we further explored the promoting effect of KIFC1 on tumor growth and whether KIFC1 participated in the process of CDK1 on tumorigenesis. Results in **Fig. 4B-D** showed that overexpression of KIFC1 in Ishikawa cells individually enhanced cell survival, proliferation, migration, and invasion, similar to the results of CDK1 overexpression in HEC-1A cells. Not surprisingly, the effect of KIFC1 overexpression in Ishikawa cells was significantly inhibited by CDK1 knockdown, including reduced cell viability, proliferation, migration, and invasion (**Fig. 4B-D**). In addition, Ishikawa cells transfected with shCDK1 and oeKIFC1 vectors significantly reduced the protein levels of CDK1 and p-KIFC1 compared to the other 2 groups. In contrast, cells transfected with shNC and oeKIFC1 vectors markedly increased the protein level of KIFC1 (**Fig. S1E**). These findings inferred that CDK1-mediated KIFC1 participation regulated cell growth and proliferation in vitro.

4. CDK1 contributes to the phosphorylation of KIFC1 to activate the PI3K/AKT signaling pathway

Based on the above results that CDK1 contributed to the phosphorylation of KIFC1, we further investigated how CDK1 phosphorylated KIFC1 in vitro. Co-IP assay referred that CDK1 interacted with KIFC1 in HEK 293T cells (**Fig. 5A**). Previous literature has reported that the phosphorylation of KIFC1 resulted in increased stability of KIFC1 and might be a specific biomarker for DNA damage [10]. The PI3K/AKT signaling pathway plays an essential role in developing EMCA by regulating tumor cell metastasis, proliferation, invasion, and cell cycle arrest [5,21]. Our previous study showed that KIFC1 activated PI3K/AKT signaling pathway to promote the migration and invasion of EMCA cells [1]. Therefore, we next investigated the effect of CDK1 on regulating the PI3K/AKT signaling pathway. As shown in **Fig. 5C**, CDK1 knockdown in Ishikawa cells dramatically dropped p-PI3K/PI3K and p-AKT/AKT protein levels. Inversely, overexpression of CDK1 in HEC-1A cells increased p-PI3K and p-AKT levels to induce further the activation of the PI3K/AKT signaling pathway (**Fig. 5B**). Moreover, we

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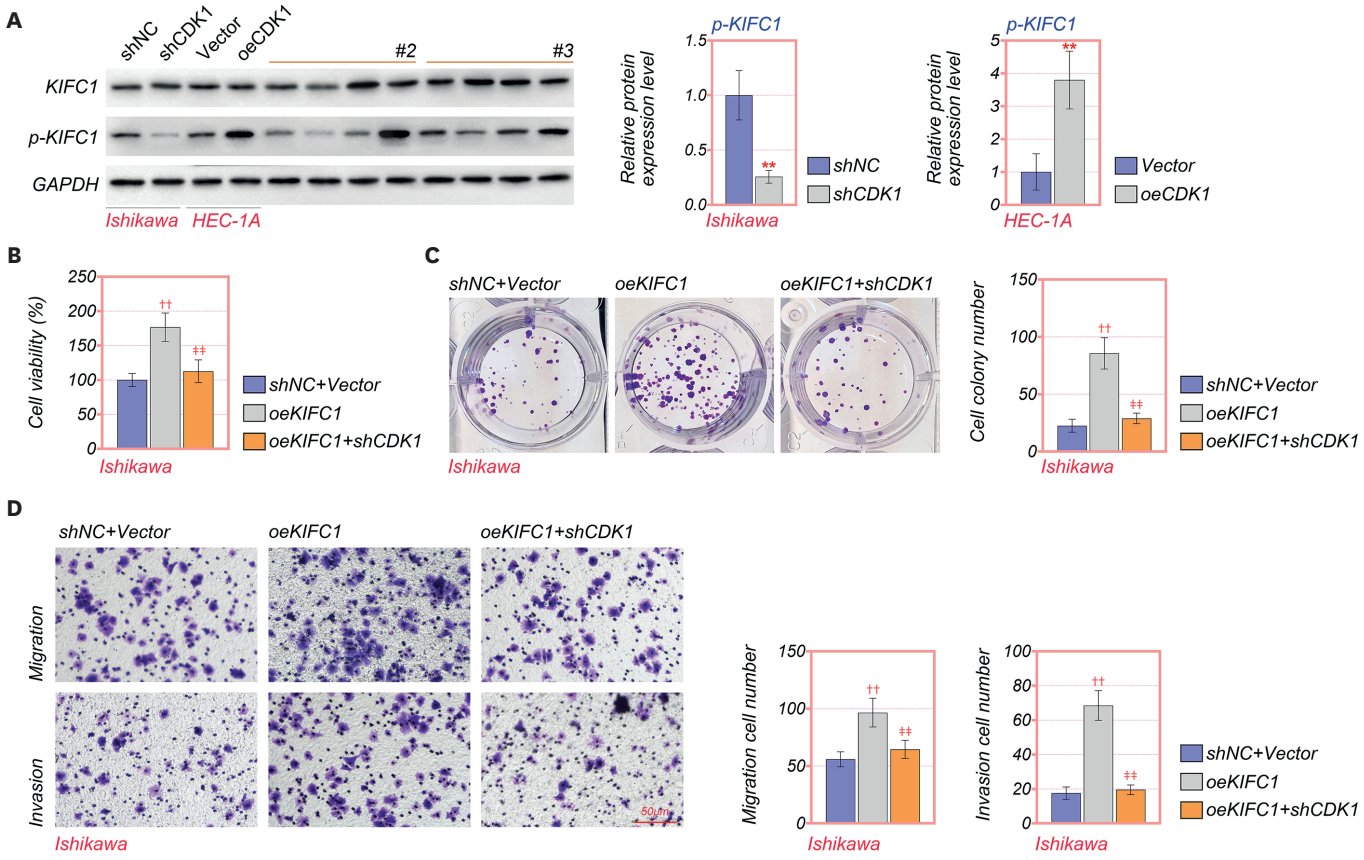

Fig. 4. CDK1 regulates the growth and proliferation of EMCA cells dependent on KIFC1 in vitro. (A) Western blotting analysis of KIFC1 and p-KIFC1 in Ishikawa cells with CDK1 knockdown and HEC-1A cells with CDK1 overexpression. (B) The cell viability of Ishikawa cells with CDK1 knockdown and KIFC1 overexpression was accessed by Cell Counting Kit-8 assay. (C) Representative images and quantification of colonies formed by Ishikawa cells with CDK1 knockdown and KIFC1 overexpression. (D) Representative images and quantification of cell migration and invasion by Transwell assays in Ishikawa cells with CDK1 knockdown and KIFC1 overexpression (scale bar: 50 μm). #2 and #3 were repetitive experiments.
CDK1, cyclin-dependent protein kinase 1; EMCA, endometrial carcinoma; GAPDH, glyceraldehyde 3-phosphate dehydrogenase; KIFC1, kinesin family member C1; oeCDK1, pcDNA3.1-cyclin-dependent protein kinase 1; oeKIFC1, pcDNA3.1-kinesin family member C1; shCDK1, short hairpin cyclin-dependent protein kinase 1; shNC, short hairpin RNA negative control.
The comparison between 2 groups: $^{**}p<0.01$.
The significance of oeKIFC1 compared to shNC + Vector: $^{††}p<0.01$.
The significance of oeKIFC1 + shCDK1 compared to oeKIFC1: $^{‡‡}p<0.01$.

also investigated the effect of KIFC1, downstream of CDK1, on PI3K/AKT pathway. Results in **Fig. 5D** showed that overexpression of KIFC1 in Ishikawa cells individually enhanced the protein levels of p-PI3K/PI3K and p-AKT/AKT, similar to the results of CDK1 overexpression in HEC-1A cells (**Fig. 5B**). Not surprisingly, transfected with shCDK1 vector in Ishikawa cells significantly inhibited the PI3K/AKT signaling pathway activated by oeKIFC1 plasmids (**Fig. 5C**). Therefore, these results inferred that CDK1 knockdown blocked the activation of the PI3K/AKT signaling pathway induced by KIFC1 overexpression. The above results suggested that PI3K/AKT signaling pathway might participate in CDK1-mediated EMCA cell survival, proliferation, invasion, migration, apoptosis, and cell cycle arrest dependent on KIFC1 phosphorylation.

5. Knockdown of CDK1 suppressed tumor formation in vivo

We finally verified whether CDK1 influenced tumorigenesis and metastasis of EMCA in vivo. Ishikawa cells transfected with shNC or shCDK1 were xenografted in nude mice by subcutaneous injection. After implantation, the tumors were excised, and the representative images were shown in **Fig. 6A**. Compared with the shNC group, the tumors in the shCDK1

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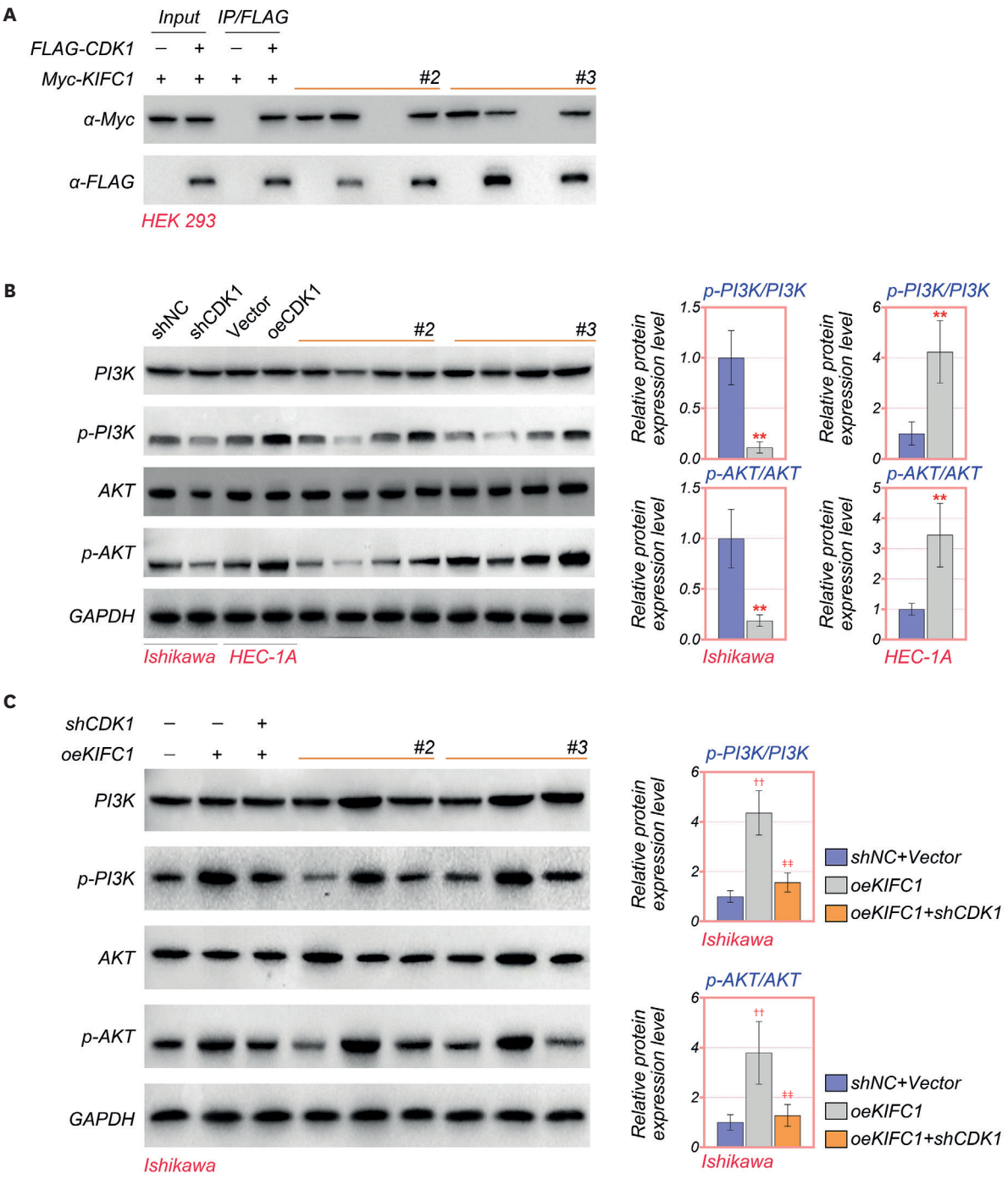

Fig. 5. CDK1 contributes to the phosphorylation of KIFC1 to activate the PI3K/AKT signaling pathway. (A) Co-immunoprecipitation was conducted with antibodies against Flag or Myc. The samples were analyzed by western blotting assay using the indicated antibodies. (B) Western blotting analysis of proteins, including PI3K, p-PI3K, AKT, p-AKT, and GAPDH in Ishikawa cells with CDK1 knockdown and HEC-1A cells with CDK1 overexpression. (C) Western blotting analysis of proteins, including PI3K, p-PI3K, AKT, p-AKT, and GAPDH in Ishikawa cells with shCDK1 and oeKIFC1 plasmids. #2 and #3 were repetitive experiments.
CDK1, cyclin-dependent protein kinase 1; GAPDH, glyceraldehyde 3-phosphate dehydrogenase; KIFC1, kinesin family member C1; oeCDK1, pcDNA3.1-cyclin-dependent protein kinase 1; oeKIFC1, pcDNA3.1-kinesin family member C1; shCDK1, short hairpin cyclin-dependent protein kinase 1; shNC, short hairpin RNA negative control.
The comparison between 2 groups: **p<0.01.
The significance of oeKIFC1 compared to shNC + Vector: ††p<0.01.
The significance of oeKIFC1 + shCDK1 compared to oeKIFC1: ‡‡p<0.01.

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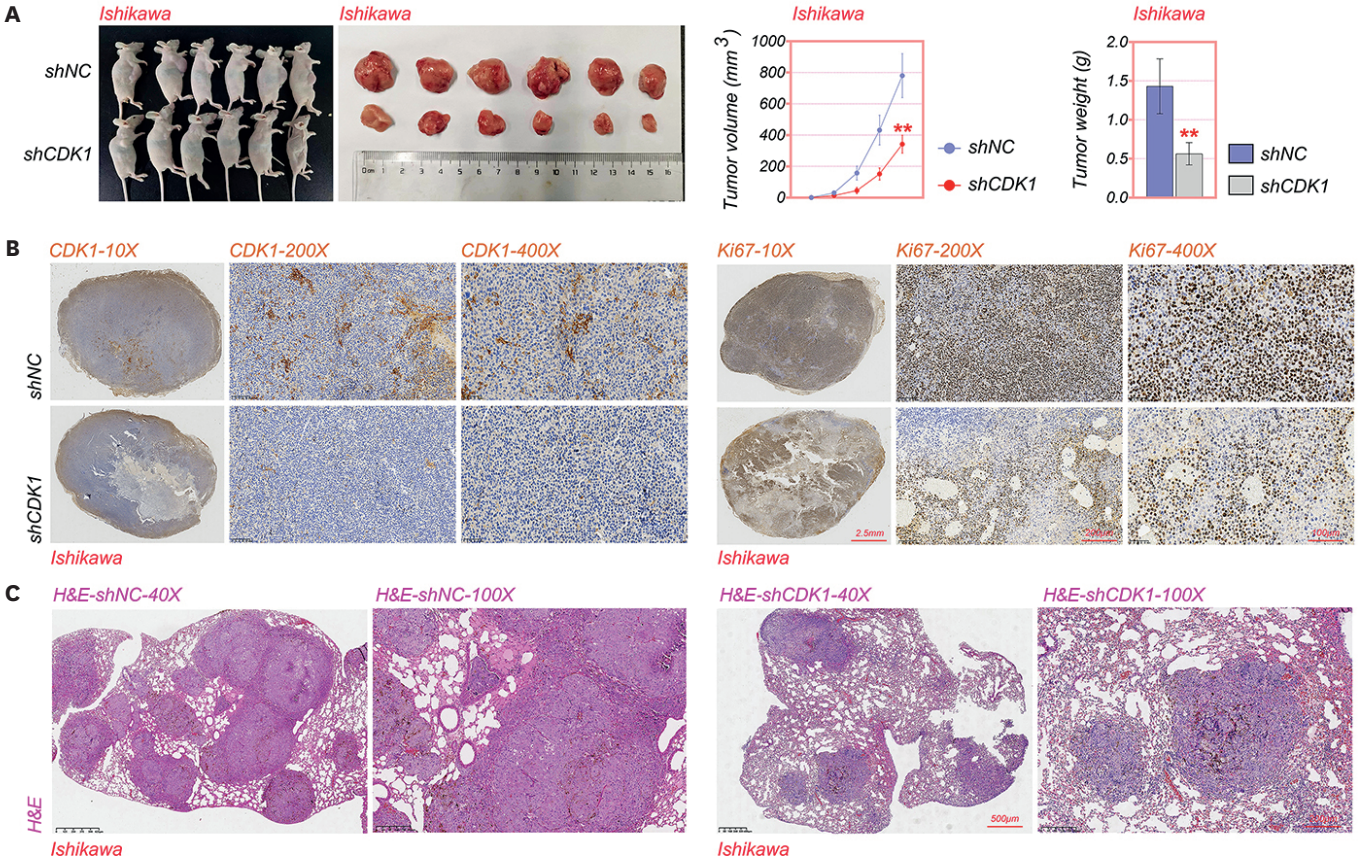

Fig. 6. Knockdown of CDK1 suppressed tumor formation in vivo. (A) CDK1 knockdown attenuated tumor growth in nude mice as indicated by tumor images (left), tumor volumes (middle), and tumor weight at surgical isolation (right). (B) Representative immunohistochemistry staining images of CDK1 and Ki67 in xenograft tumors (scale bar: 2.5 mm for 10×; 200 μm for 200×; 100 μm for 400×). (C) Representative H&E staining images of lung tissues (scale bar: 500 μm for 40×; 200 μm for 100×). CDK1, cyclin-dependent protein kinase 1; H&E, hematoxylin and eosin; shCDK1, short hairpin cyclin-dependent protein kinase 1; shNC, short hairpin RNA negative control. The comparison between 2 groups: **p<0.01.

group expressed smaller volumes and lighter weights (**Fig. 6A**). Additionally, IHC staining showed that CDK1 knockdown decreased the expression of CDK1, KIFC1, and Ki-67 in xenograft tumors (**Fig. 6B**, **Fig. S1F**), and H&E staining indicated that CDK1 knockdown inhibited severe histological damage of lung tissues (**Fig. 6C**). These results demonstrated that CDK1 knockdown suppressed the tumor growth and metastasis of EMCA in vivo.

DISCUSSION

EMCA is one of the most diagnosed and prevalent cancers worldwide, especially for women in the developed world [3]. Many EMCA patients have been cured with surgical resection and postoperative chemoradiotherapy [1]. However, high invasion and metastasis of tumor cells led to poor survival and prognosis. Therefore, a better understanding of the pathogenesis and molecular mechanism of EMCA is necessary, and finding effective therapeutic targets is of great importance. Previous studies have reported that CDK1, an essential cell cycle regulator, promoted the progression of different cancers, such as lung cancer [8,9,22]. However, the concrete role of CDK1 in the pathogenesis of EMCA was vague and needed further investigation. The primary purpose of our project was to clarify the regulatory effect and potential mechanism of CDK1 in the development of EMCA in vivo and in vitro.

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Kinds of literature have inferred that CDK1 was highly enriched in various cancers, including endometrioid endometrial [2,23] and hepatocellular carcinoma [24]. Similarly, our results showed an over-expression of CDK1 in EMCA patients and various tumor cell lines. Subsequently, we clarified the effect of CDK1 in the progression of EMCA. Uncontrolled cell growth, invasion, and metastasis are significant characteristics and contributors to various cancers [6,9,22], including EMCA [3]. A previous study reported that CDK1 regulated cell proliferation, apoptosis, and migration to promote the progression of colorectal cancer [25,26]. Another recent study also demonstrated that overexpression of CDK1 in adrenocortical carcinoma (ACC) cell lines promoted proliferation and induced the epithelial-to-mesenchymal transition, whereas silencing of CDK1 and CDK1 inhibitor suppressed the growth of ACC cell lines [26]. In our study, CDK1 knockdown inhibited cell viability, proliferation, invasion, and migration and promoted apoptosis in Ishikawa and HEC-1B cells. The above results were further verified by CDK1 overexpression in HEC-1A cells. Together, these observations indicated that CDK1 facilitated the progression of EMCA.

Previous studies have inferred that aberrancy in cell cycle progression and DNA damage are the fundamental mechanisms underlying different tumorigenesis [27,28]. CDK1, belonging to the CDKs family, is vital in initiating mitosis and regulating different phases in the mammalian cell cycle [7,24]. Moreover, CDK1 was reported to regulate the G2/M phase transition in ACC cells [26]. In this study, CDK1 knockdown in Ishikawa cells markedly dropped the level of γH2AX, while overexpression of CDK1 contributed to the accumulation of γH2AX in HEC-1A cells. γH2AX, a novel biomarker of DNA double-stranded breaks, is used to monitor DNA damage and repair [29]. Positive staining for γH2AX indicated genomic instability and telomere dysfunction and was usually observed in tumor cells and tissues [30]. Our findings suggested that CDK1 led to DNA damage similar to others' studies [31]. Moreover, CDK1, a regulator of S/G2 and G2/M transitions, participated in the cell cycle process in different tumor models [7,26]. Previous studies have reported that CDK1 inhibitor caused G2/M phase arrest in human glioblastoma cells and endometrial cancer cells [2,32], and the interaction between centromere protein F and CDK1 augmented the G2/M phase transition of mitosis and cell proliferation in human ACC [33]. Not surprisingly, CDK1 knockdown in Ishikawa cells induced an increased proportion of cells in the G2/M phase, and CDK1 overexpression in HEC-1A cells promoted the cell cycle progression in this study. These results further indicated that CDK1 contributed to DNA damage and cell cycle arrest to promote the pathogenesis of EMCA.

Posttranslational modification is critical for various proteins to take effect in cellular functions and activities. One previous study demonstrated that phosphorylated KIFC1 at Ser6 by CDK1 positively impacted stabilizing the protein level of KIFC1 [34]. KIFC1 was also found to participate in the pathogenesis and development of various cancers [6,14], including EMCA [1]. Our study showed that KIFC1 was highly expressed in tissues of EMCA patients, and CDK1 regulated the growth and proliferation of EMCA cells dependent on KIFC1 phosphorylation. In addition, phosphorylated KIFC1 was required for the process. PI3K/AKT signaling pathway regulates cancer development [5,21]. At the same time, KIFC1 overexpression activated PI3K/AKT signaling pathway by increasing the protein levels of p-PI3K and p-AKT in our previous study [1]. In this work, results inferred that CDK1 knockdown in Ishikawa cells also inhibited the activation of PI3K/AKT induced by KIFC1 overexpression. Together, CDK1 promoted cell growth and migration of EMCA tumor cells via KIFC1-mediated PI3K/AKT signaling pathway activation.

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Finally, a xenograft mouse model proved the anticancer effect of CDK1 inhibition on tumorgenicity and metastasis. Other studies have indicated that CDK1 inhibitor RO3306 inhibited the growth of HEC-1B cells [2], and CurE, another CDK1 inhibitor, dropped the proliferation of ACC cells in vitro and in vivo [26]. Consistently, our results also showed that the knockdown of CDK1 in Ishikawa cells inhibited tumor growth, proliferation, and metastasis in vivo and in vitro.

In conclusion, our finding firstly demonstrated the promoting effect of CDK1 on EMCA progression by targeting the KIFC-mediated PI3K/AKT signaling pathway to increase cell growth, metastasis, and DNA damage and restrict cell apoptosis and cell cycle (**Fig. S2**), which indicated the oncogenic ability of CDK1. Although the potential mechanism needs future investigation, targeting CDK1 may be a promising treatment for EMCA.

SUPPLEMENTARY MATERIALS

Table S1
Detailed patient characteristics

Fig. S1
KIFC1 expression and its impact on cell functions. (A) Representative H&E and immunohistochemistry images of KIFC1 in paired normal tissues and endometrial carcinoma tissues (scale bar: 200 μm for 200×; 100 μm for 400×). (B) The transfection efficiency of CDK1 knockdown in HEC-1B cells was evaluated by quantitative polymerase chain reaction analysis. (C) Representative images and quantification of colonies formed by HEC-1B cells with CDK1 knockdown. (D) Representative images and quantification of cell migration and invasion by Transwell assays in HEC-1B cells with CDK1 knockdown (scale bar: 50 μm). (E) Western blotting analysis of proteins, including CDK1, KIFC1, p-KIFC1, and GAPDH in Ishikawa cells with shCDK1 and oeKIFC1 plasmids. (F) Representative IHC staining images of KIFC1 in xenograft tumors (scale bar: 100 μm for 400×).

Fig. S2
Schematic diagram. In our study, CDK1 was found to be highly expressed in EMCA patients and tumor cells, leading to increased cell survival, proliferation, migration, invasion, DNA damage, and decreased cell apoptosis. Mechanically, CDK1 promoted the phosphorylation of KIFC1, which activated the PI3K/AKT signaling pathway to influence the cell phenotypes.

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