BMC Cancer

RESEARCH Open Access

Deubiquitinase USP18 mediates cell migration, apoptosis and ferroptosis in lung adenocarcinoma by depending on POU4F1/PRKAA2 axis

Xinping Pan[1] and Hui Deng[2*]

Abstract

Background Lung adenocarcinoma (LUAD) is a common type of lung cancer and its pathogenic mechanism is complicated. A profound research for the molecular mechanism in LUAD is indispensable.

Methods Gene levels were detected via real-time quantitative polymerase chain reaction and western blot. Proliferation, migration and apoptosis were assessed using colony formation assay, wound healing assay, and flow cytometry. Ferroptosis was evaluated through oxidative stress and iron level. Relations between genes were analyzed using Immunoprecipitation (IP) assay and ubiquitination assay, as well as ChIP assay and dual-luciferase reporter assay. USP18 function in vivo was explored using xenograft model.

Results Ubiquitin-specific protease 18 (USP18) was overexpressed in LUAD tissues and cells. LUAD cell proliferation and migration were suppressed but apoptosis and ferroptosis were enhanced after USP18 knockdown. Pou domain, class 4, transcription factor 1 (POU4F1) protein expression was stabilized through USP18-mediated deubiquitination. Function of USP18 silence was reversed by POU4F1 overexpression in LUAD cells. POU4F1 promoted transcription of AMPK-α2 (PRKAA2) and USP18 modulated PRKAA2 protein level via affecting POU4F1. POU4F1 regulated LUAD cell behaviors by upregulating PRKAA2. USP18 enhanced tumor growth in vivo via mediating POU4F1 and PRKAA2.

Conclusion All data demonstrated that USP18 acted as an oncogene in LUAD via interacting with POU4F1/PRKAA2 axis.

Highlights

- Silence of USP18 promotes lung adenocarcinoma cell apoptosis and ferroptosis.
- USP18 affects lung adenocarcinoma cell progression by stabilizing POU4F1 protein.
- USP18 regulates PRKAA2 via enhancing POU4F1.

Keywords USP18, POU4F1, PRKAA2, Lung adenocarcinoma

*Correspondence:
Hui Deng
18054160605@163.com
[1]Department of Radiotherapy, Dingxi People's Hospital, Dingxi City 743000, Gansu Province, PR China
[2]Department of Oncology, Dingxi People's Hospital, Jiangtai Street, Anding District, Dingxi City 743000, Gansu Province, PR China

Introduction

Lung adenocarcinoma (LUAD) is a common type of lung cancer and it accounts for about 40% of cases in lung cancers [1]. LUAD poses a serious global health burden due to high morbidity and frequent recurrence or drug resistance, ultimately resulting in unfavorable prognosis [2, 3]. It is imperative to comprehend the biological mechanisms underlying tumor malignant development to discover the advanced treatment strategies of LUAD. Ferroptosis is an iron-dependent regulated cell death (RCD) caused by lipid peroxidation, and targeting ferroptosis is considered as an alternative therapy for targeting apoptosis in LUAD [4]. Hence, discovering the biomarkers for ferroptosis may contribute to the treatment of LUAD.

Deubiquitination is a pivotal post-translational modification in the protein reactions of human body and plays an essential role in multiple diseases [5]. Deubiquitinating enzymes (DUBs) are vital regulators of deubiquitination by removing ubiquitin (Ub) molecules from various substrates and maintaining protein stability [6]. DUBs have been regarded as anticancer target candidates for LUAD treatment [7]. Ubiquitin-specific proteases (USPs) are one of the common types of DUBs, and exhibit the pivotal regulation in LUAD via the mediation of deubiquitination. For example, USP22 facilitated oncogenic activity of LUAD cells by acting as a DUB of COL17A1 [8]. Also, USP38 exerted the promoting function in the malignant progression for LUAD by enhancing the deubiquitination of KLF5 [9]. USP18 is a common DUB with a central role in human cancer [10]. The functional role and regulatory mechanism of USP18 in LUAD remain to be investigated.

USP18 is known to regulate various processes to accelerate the development of several autoimmune diseases and carcinogenesis [11]. USP18 can stabilize Twist1 by cleaving its ubiquitination to facilitate cell metastasis in glioblastoma [12]. USP18 was shown to promote lung tumorigenesis and affect fatty acid metabolism [13]. In addition, loss of USP18 suppressed lung cancer cell metastasis by destabilizing the downstream target [14]. To a certain extent, the oncogenic function of USP18 in lung cancer is attributed to the deubiquitination regulation. DUBs can regulate the main types of RCD, including apoptosis and ferroptosis [15]. The effects of USP18 on LUAD cellular behaviors, especially in ferroptosis, are worth studying.

Pou domain, class 4, transcription factor 1 (POU4F1) is a member of class IV POU genes with unique upregulation and tumorigenic properties in many cancers, such as breast cancer and esophageal squamous cell carcinoma [16, 17]. POU4F1 was reported to be aberrantly expressed in lung cancer [18], however, the role of POU4F1 in LUAD is still unexplored. AMPK-α2 (PRKAA2) is an AMP-activated protein kinase with oncogenic regulation in promoting invasion and migration of LUAD [19]. In addition, PRKAA2 mediated by miR-186-5p could affect ferroptosis of LUAD cells [19]. The potential deubiquitination of USP18 for POU4F1, as well as the relation between POU4F1 and PRKAA2, is completely unknown in LUAD. Meanwhile, whether the effects of USP18 and POU4F1 on ferroptosis are related to PRKAA2 is required to be explored.

In this study, the biological function and deubiquitination effect of USP18 were mainly explored. Moreover, the transcriptional regulation of POU4F1 on PRKAA2 was also studied. USP18-mediated POU4F1/PRKAA2 axis was firstly investigated in migration and ferroptosis of LUAD.

Materials and methods

Datasets

Expression prediction was administrated via Timer (https://cistrome.shinyapps.io/timer/) and Ualcan (https://ualcan.path.uab.edu/index.html). Ubibrowse (http://ubibrowser.bio-it.cn/ubibrowser/) and Jaspar (https://jaspar.elixir.no/analysis) were employed for predicting gene interaction.

Tissues and cells

LUAD samples and normal controls were acquired from LUAD patients ($n = 35$) with surgical treatment at Dingxi People's Hospital, then instantly stored at -80℃. Gene protein expression in tissue samples was analyzed using immunohistochemistry (IHC) as previously reported [20]. All procedures were authorized by the Ethics Committee of Dingxi People's Hospital, based on acquisition of the written informed consent forms. LUAD cells (H1299, A549) and normal 16HBE cells (BioVector NTCC Inc., Beijing, China) were grown in culture solution consisted of DMEM medium, 10% fetal bovine serum and 1% antibiotic in 37℃, 5% CO_2 incubator. These reagents for cell culture were provided by Gibco (Carlsbad, CA, USA).

Transfection of RNA or vector

Short hairpin RNA against USP18 (sh-USP18) or POU4F1 (sh-POU4F1), pcDNA-USP18 (USP18), pcDNA-POU4F1 (POU4F1) and pcDNA-PRKAA2 (PRKAA2) were purchased from GENESEED (Guangzhou, China). Normal control (NC) was set by sh-NC and pcDNA. Transfection of these RNAs and vectors was implemented utilizing Lipofectamine™ 3000 (Invitrogen, Carlsbad, CA, USA), then cells were collected for the later research.

Real-time quantitative polymerase chain reaction (RT-qPCR)

Total RNA was separated from clinical samples or harvested cells employing RNAiso Plus Reagent (Takara, Beijing, China), followed by transformation of RNA into cDNA via PrimeScript™ RT reagent Kit (Takara) and cDNA amplification through TB Green®*Premix Ex Taq*™ II reagent (Takara) applying with specific primers (Table 1). Gene expression was standardized to β-actin, and data analysis was administrated via $2^{-\Delta\Delta Ct}$ method.

Western blot

After total protein extraction by RIPA buffer (Sangon, Shanghai, China), 50 μg proteins of each group were electrophoresed. Isolated proteins on gels were transferred onto PVDF membranes (Sigma, St. Louis, MO, USA), and then blocked in 5% non-fat milk (Invitrogen). PVDF membranes and the primary antibodies against USP18 (Abcam, Cambridge, UK; ab161390), POU4F1 (Abcam, ab245230), PRKAA2 (Abcam, ab214425) were co-incubated at 4℃ overnight with a ratio of 1:1000. β-actin (Abcam, ab8227) was employed as the internal control. After the secondary antibody (Abcam, ab205718, 1:2000) was incubated at 25℃ for 45 min, protein blots were displayed via Electrochemiluminescence (ECL) regent (Sigma). Image J software was utilized for protein level analysis.

Colony formation assay

LUAD with 500 cells/well in 6-well plates were cultivated under normal condition for 14 d. When the white colonies were observed, methanol (Beyotime, Shanghai, China) and crystal violet (Beyotime) were added to fixate and stain these colonies. Finally, colonies were numbed using Image J software.

Wound healing assay

LUAD cells were cultured to mono-layer confluence, then two straight scratches were generated employing a sterile pipette tip. Cells of each well were washed and then cultured with serum-free medium for 24 h. Images were obtained by a digital camera. Wound healing ratio = wound distance/wound $width_{0\,h}$.

Table 1 Primer sequences used for RT-qPCR

Name	Primer sequences (5′-3′)	
USP18	Forward	AGTGAAGTCGTGCTGTCCTG
	Reverse	CCCAAACGCCTTGCTCATTC
POU4F1	Forward	TGCATGTTGGGCGAAAACAG
	Reverse	TCCACCAGCAGCTTGTAAGG
PRKAA2	Forward	CGGGTGAAGATCGGACACTA
	Reverse	AACTGCCACTTTATGGCCTG
β-actin	Forward	CTTCGCGGGCGACGAT
	Reverse	CCACATAGGAATCCTTCTGACC

Flow cytometric assay

Firstly, 4×10^4 cells were harvested after 48 h of transfection. Subsequently, cells were incubated with Annexin V-FITC and Propidium Iodide in accordance with operation procedures of Annexin V-FITC Apoptosis Detection Kit (Invitrogen). Then, Flow cytometer (BD Biosciences, San Diego, CA, USA) was utilized for cell analysis. Apoptosis rate = apoptotic cells/total cells ×100%.

Oxidative analysis

Reactive oxygen species (ROS), malondialdehyde (MDA) and glutathione (GSH) were examined through ROS Assay Kit (Invitrogen), MDA Assay Kit (Sigma) and Glutathione Colorimetric Detection Kit (Invitrogen) based on the users' guidelines.

Fe^{2+} level

Fe^{2+} level was determined via iron assay kit (Sigma) in line with the supplied specification. After Fe^{3+} was reduced to Fe^{2+} by Iron Reducer, iron Probe was incubated for 1 h away from light and absorbance at 593 nm was examined.

Immunoprecipitation (IP) assay

H1299 and A549 cells were lysed in RIPA containing protease inhibitor (Beyotime), followed by concentration detection via BCA protein assay kit (Beyotime). Cell lysates were immunoprecipitated with antibodies overnight at 4℃, followed by incubation with protein A/G-agarose beads (Beyotime). The complexes were washed five times, then the bound proteins were collected for western blot analysis.

Ubiquitination assay

H1299 and A549 cells were performed with transfection of sh-NC and sh-USP18 for 24 h. Then the immunoprecipitated protein was washed with lysis buffer and then examined by western blot with anti-USP18 (Abcam, ab161390), anti-POU4F1 (Abcam, ab245230), and anti-ubiquitin (Abcam, ab7780) antibodies. In addition, cells were transfected with pcDNA, USP18, or enzyme-dead USP18-C64S mutant followed by ubiquitination level detection of POU4F1.

Protein stability detection

H1299 and A549 cells were transfected with sh-NC or sh-USP18, followed by treatment with 100 μM cyclohexylamine (CHX, Sigma) for different times (0 h, 3 h, 6 h, 9 h and 12 h) or 20 μM MG132 (Selleck, Houston, TX, USA) for 3 h. Then the protein level of POU4F1 was detected using western blot. Additionally, half-life of POU4F1 was detected by western blot after transfection with USP18 or USP18-C64S under the treatment of CHX.

ChIP

Enrichment of PRKAA2 promoter was evaluated through using a ChIP Assay Kit (Beyotime) following users' guidelines. POU4F1 antibody (anti-POU4F1) or IgG antibody (anti-IgG) was mixed with clear nuclear lysates for IP. Co-precipitated DNA was purified and enrichment of PRKAA2 promoter was assayed via qPCR.

Dual-luciferase reporter assay

PRKAA2 sequences of wild-type (WT) and mutant-type (MUT) were cloned into the empty pmirGLO plasmid (Promega, Madison, WI, USA), respectively. PRKAA2-WT or PRKAA2-MUT and pcDNA or POU4F1 were co-transfected into H1299 and A549 cells. Following 48 h, cells were harvested and then luciferase level was evaluated via Dual-Luciferase® Reporter Assay System (Promega).

Animal model

Six-week-old male BALB/c nude mice (Vital River Laboratory Animal Technology, Beijing, China) were employed for establishing xenograft model. 5×10^6 A549 cells with different transfection (sh-NC, sh-USP18, sh-USP18 + POU4F1, sh-POU4F1, or sh-POU4F1 + PRKAA2) were subcutaneously injected into the right of back of mice. Tumor growth was observed via recording volume (length × width^2/2) and mice was euthanized at 28 d. CO_2 inhalation is currently the most common method of euthanasia for laboratory mice [21]. All mice were sacrificed by displacing the 30% air of cage each minute using the flow rate of CO_2 according to the current guideline of American Veterinary Medical Association (AVMA). Then, tumors were dissected and weight of each tumor was measured. PRKAA2 protein detection was implemented through western blot and IHC assay. All operations on mice have obtained ratification from the Animal Ethical Committee of Dingxi People's Hospital.

Statistical analysis

Group comparison was administrated through Student's *t*-test or ANOVA followed by Tukey's test. Each experiment was independently carried out for three times. Data were revealed as mean ± SD. SPSS 22.0 and GraphPad Prism were applied for statistical analysis. $P < 0.05$ was shown as a significant difference.

Results

USP18 was upregulated in LUAD tissues and cells

Timer dataset (Fig. 1A) and Ualcan dataset (Fig. 1B) predicted that USP18 was highly expressed in LUAD tumor samples. After tissue collection, IHC analysis demonstrated that USP18 protein expression was upregulated in LUAD compared to normal tissues (Fig. 1C). RT-qPCR and western blot results exhibited the upregulation of USP18 in LUAD tissues relative to non-cancer tissues (Fig. 1D-E). Also, mRNA and protein levels of USP18 were significantly increased in H1299 and A549 cells contrasted with normal 16HBE cells (Fig. 1F-G). USP18 was confirmed as a upregulated gene in LUAD.

Knockdown of USP18 promoted apoptosis and ferroptosis of LUAD cells

H2199 and A549 cells were transfected with sh-NC, sh-USP18 for function analysis of USP18. Western blot indicated that knockdown efficiency of sh-USP18 was significant when compared to sh-NC group (Fig. 2A). By performing colony formation assay, colony number was shown to be decreased after silence of USP18 (Fig. 2B). Wound healing assay suggested that cell migration ability was suppressed, as the result of USP18 downregulation (Fig. 2C). More importantly, RCD plays a essential role in cancer progression, and we further explored the effects of USP18 on cell apoptosis and ferroptosis. Apoptosis rate (Fig. 2D) and ROS level (Fig. 2E) were increased after USP18 was knocked down. Silencing USP18 resulted in the upregulation of MDA (Fig. 2F), the reduction of GSH (Fig. 2G) and the increase of Fe^{2+} level (Fig. 2H). In summary, USP18 knockdown inhibited proliferation, migration but enhanced apoptosis and ferroptosis in LUAD cells.

USP18 stabilized POU4F1 protein expression through inducing de-ubiquitination

Ubibrowser dataset revealed that POU4F1 was a substrate of USP18 (Fig. 3A). IP assay indicated that POU4F1 could be detected by anti-USP18 and USP18 was also enriched by anti-POU4F1, suggesting the interaction between USP18 and POU4F1 in LUAD cells (Fig. 3B-C). In addition, knockdown of USP18 reduced the protein expression of POU4F1 but increased the ubiquitination level of POU4F1 (Fig. 3D). In other words, USP18 could induce de-ubiquitination and upregulate POU4F1. Then, POU4F1 protein stability was assessed after CHX treatment. CHX obviously disturbed the protein synthesis of POU4F1, and half-life of POU4F1 protein expression with CHX treatment was significantly decreased after USP18 knockdown (Fig. 3E-F). Furthermore, we found MG132 (a proteasome inhibitor) blocked the protein degradation of POU4F1 caused by silence of USP18 (Supplementary Fig. 1A-B). Moreover, enzyme-dead USP18-C64S mutant resulted in the prominent inhibition of POU4F1 protein level and it reversed USP18-induced reduction of ubiquitination level of POU4F1 (Supplementary Fig. 1C). Half-life of POU4F1 with CHX treatment was shorter in USP18-C64S group than that in USP18 group (Supplementary Fig. 1D). Therefore, USP18 could enhance protein stability of POU4F1 through the

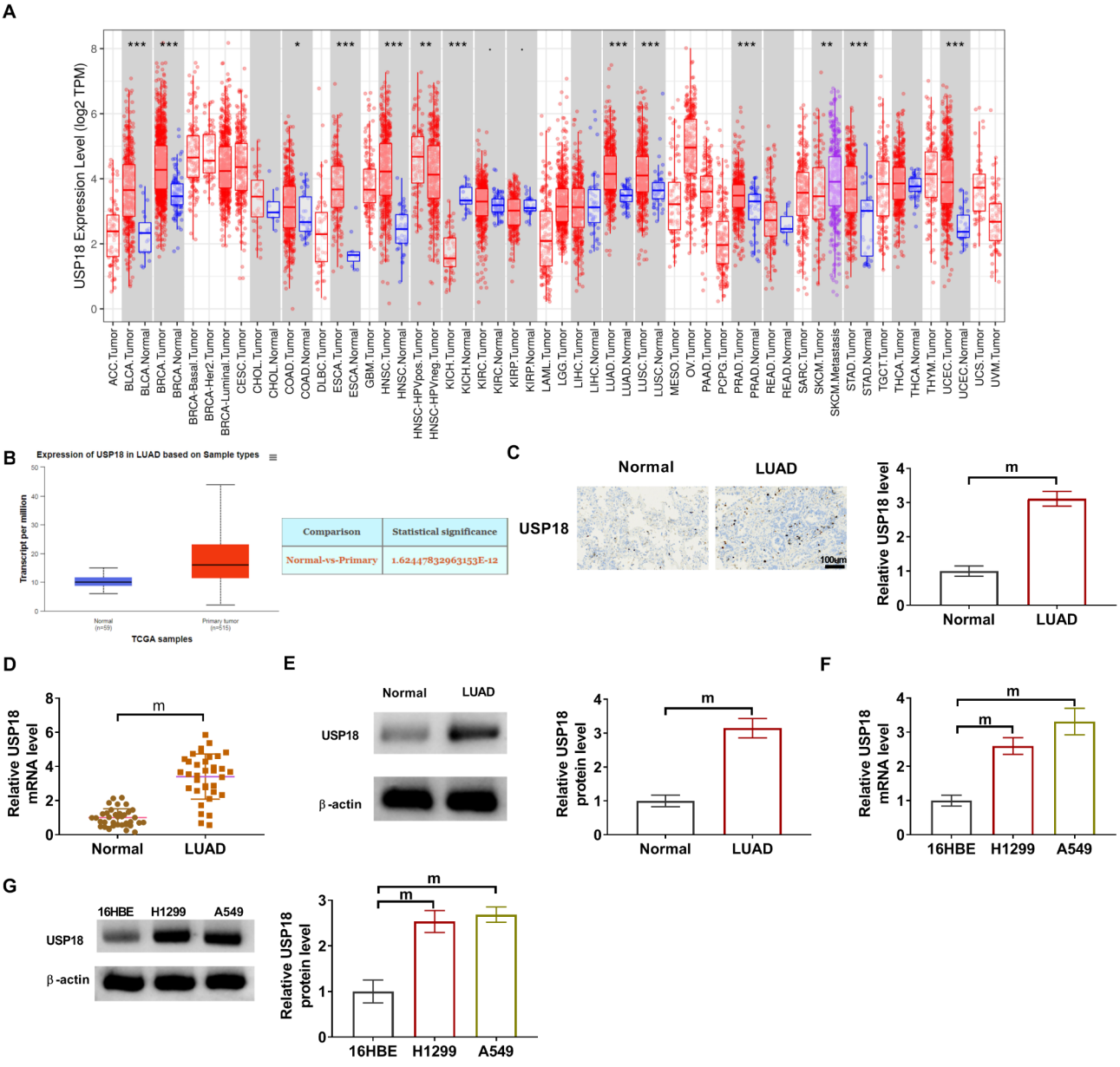

Fig. 1 USP18 was upregulated in LUAD tissues and cells. **(A-B)** USP18 expression in LUAD in Timer dataset **(A)** and Ualcan dataset **(B)**. **(C)** USP18 expression analysis in LUAD was conducted by IHC. According to the positive cells (brown), IHC quantification analysis was carried out by normalization of normal group to 1. **(D-E)** RT-qPCR **(D)** and western blot **(E)** were employed for mRNA and protein analysis in LUAD tissues. **(F-G)** RT-qPCR **(F)** and western blot **(G)** for USP18 expression detection in LUAD (H1299, A549) and normal 16HBE cells. $^{m}P < 0.001$

mediation of de-ubiquitination. In IHC assay, POU4F1 protein expression was higher in LUAD tissues relative to normal controls (Fig. 3G). Overexpression of POU4F1 was also detected in LUAD tissues through RT-qPCR and western blot assays (Fig. 3H-I). Meanwhile, POU4F1 mRNA and protein levels were distinctly increased in H1299 and A549 cells (Fig. 3J-K). Silencing USP18 reduced the protein expression of POU4F1 and this effect was then reversed by overexpression of POU4F1, which suggested the positive regulation of USP18 on POU4F1 expression (Fig. 3L). USP18 stabilized POU4F1 protein level via causing de-ubiquitination.

Anti-tumor effects of USP18 silence were rescued by overexpression of POU4F1 in LUAD cells

Analysis of colony number and wound healing ratio demonstrated that cell proliferation (Fig. 4A + Supplementary Fig. 2A) and migration (Fig. 4B + Supplementary Fig. 2B) were enhanced in sh-USP18 + POU4F1 group, relative to alone sh-USP18 group. Also, sh-USP18-induced cell apoptosis was counteracted following transfection with

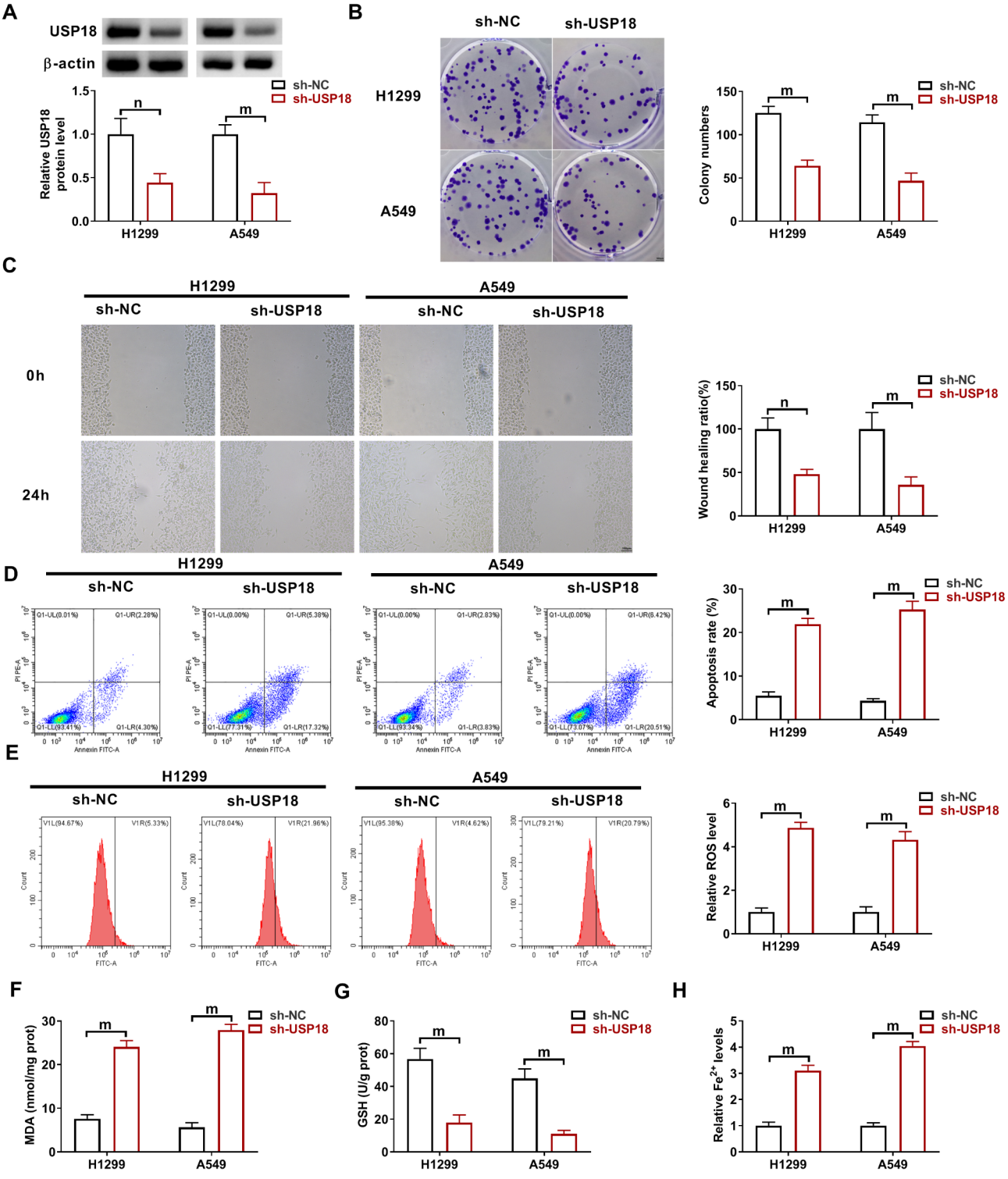

Fig. 2 Knockdown of USP18 promoted apoptosis and ferroptosis of LUAD cells. Transfection with sh-NC or sh-USP18 was performed in H1299 and A549 cells. **(A)** Analysis of USP18 protein level by western blot. **(B-C)** Cell proliferation **(B)** and migration **(C)** were assessed via colony formation assay and wound healing assay. **(D)** Apoptosis detection by flow cytometric assay. **(E-G)** ROS **(E)**, MDA **(F)**, and GSH **(G)** examination using kits. **(H)** Fe^{2+} level was determined via iron level assay. $^{n}P < 0.01$, $^{m}P < 0.001$

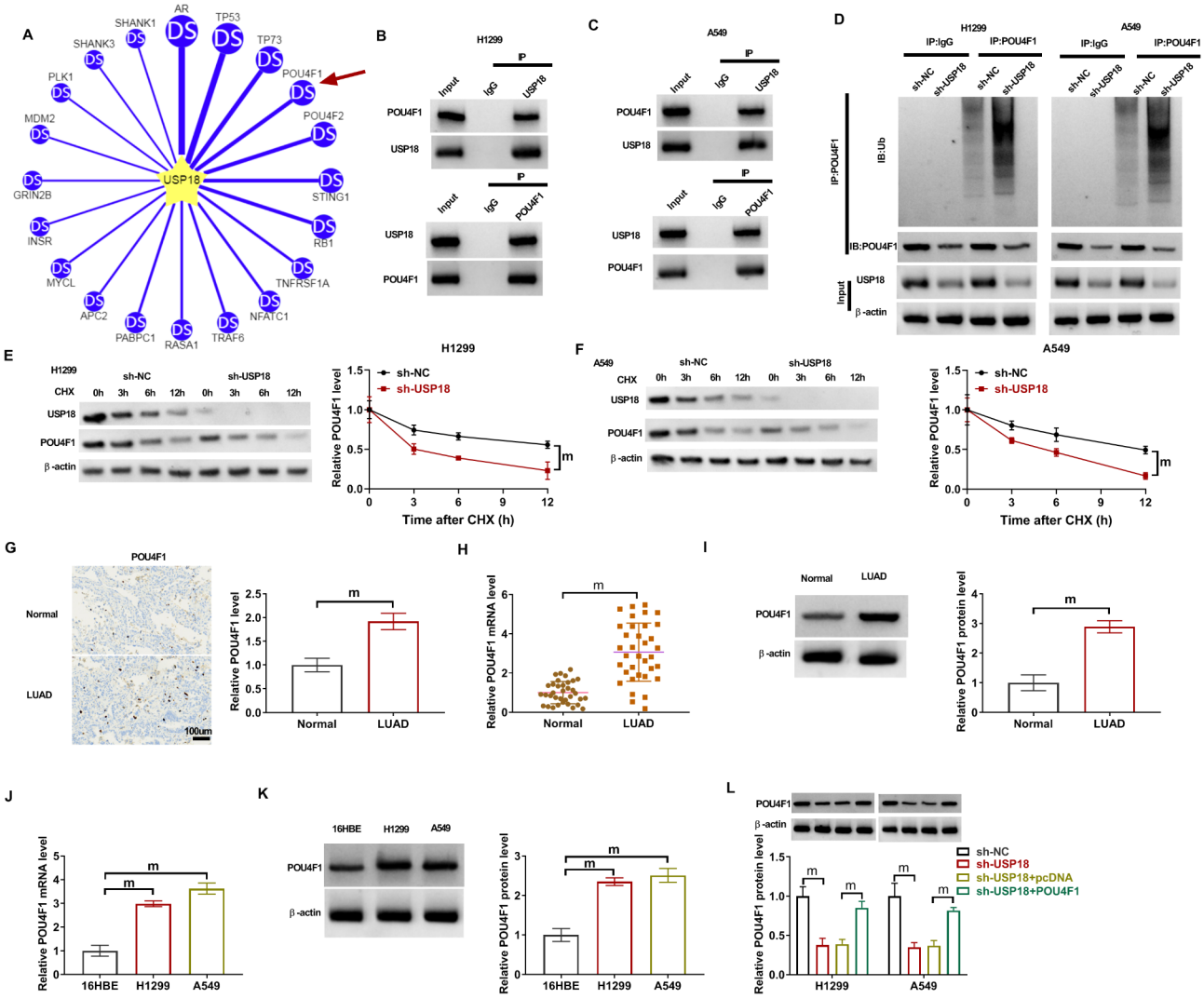

Fig. 3 USP18 stabilized POU4F1 protein expression through inducing de-ubiquitination. **(A)** POU4F1 was predicted as a USP18 substrate in Ubibrowser. **(B-C)** Analysis of USP18 and POU4F1 interaction by IP assay. **(D)** The degree of POU4F1 ubiquitination was examined using western blot after USP18 knockdown. **(E-F)** Half-life of POU4F1 with CHX treatment under knockdown of USP18 was detected by western blot. **(G)** POU4F1 protein in LUAD tissues was measured by IHC. According to the positive cells (brown), IHC quantification analysis was performed by normalization of normal group to 1. **(H-I)** RT-qPCR **(H)** and western blot **(I)** were administrated for POU4F1 detection in LUAD samples. **(J-K)** POU4F1 mRNA and protein detection by RT-qPCR and western blot in LUAD cells. **(L)** POU4F1 protein analysis by western blot after co-transfection with sh-USP18 and POU4F1, and control groups. $^{m}P < 0.001$

POU4F1 (Fig. 4C-D). Overexpression of POU4F1 alleviated the promoting regulation of sh-USP18 in ROS and MDA levels (Fig. 4E-F). GSH reduction (Fig. 4G) and Fe^{2+} level upregulation (Fig. 4H) triggered by sh-USP18 were mitigated after POU4F1 was overexpressed. The regulatory function of USP18 was achieved by mediating POU4F1 in LUAD cells.

USP18 affected PRKAA2 protein expression via deubiquitinating POU4F1 in LUAD cells

PRKAA2 was predicted to be upregulated in LUAD tissues by Timer dataset (Fig. 5A). Furthermore, high level of PRKAA2 protein was detected in LUAD samples compared with normal samples through IHC analysis (Fig. 5B). Moreover, RT-qPCR and western blot showed the aberrant overexpression of PRKAA2 in LUAD tissues (Fig. 5C-D) and cells (Fig. 5E-F). Jaspar dataset displayed that there was binding site (GAGAGGCAGA GA) between POU4F1 and PRKAA2 prompter region (Fig. 5G). ChIP assay further indicated that PRKAA2 was enriched by anti-POU4F1 relative to anti-IgG, suggesting the interaction between POU4F1 and PRKAA2 (Fig. 5H). Luciferase activity was shown to be increased after co-transfection with PRKAA2-WT and POU4F1, while no change was observed after co-transfection of PRKAA2-MUT and POU4F1 (Fig. 5I-J). Hence, POU4F1 could directly bind to PRKAA2 in LUAD cells. PRKAA2 protein expression was decreased by sh-POU4F1 and

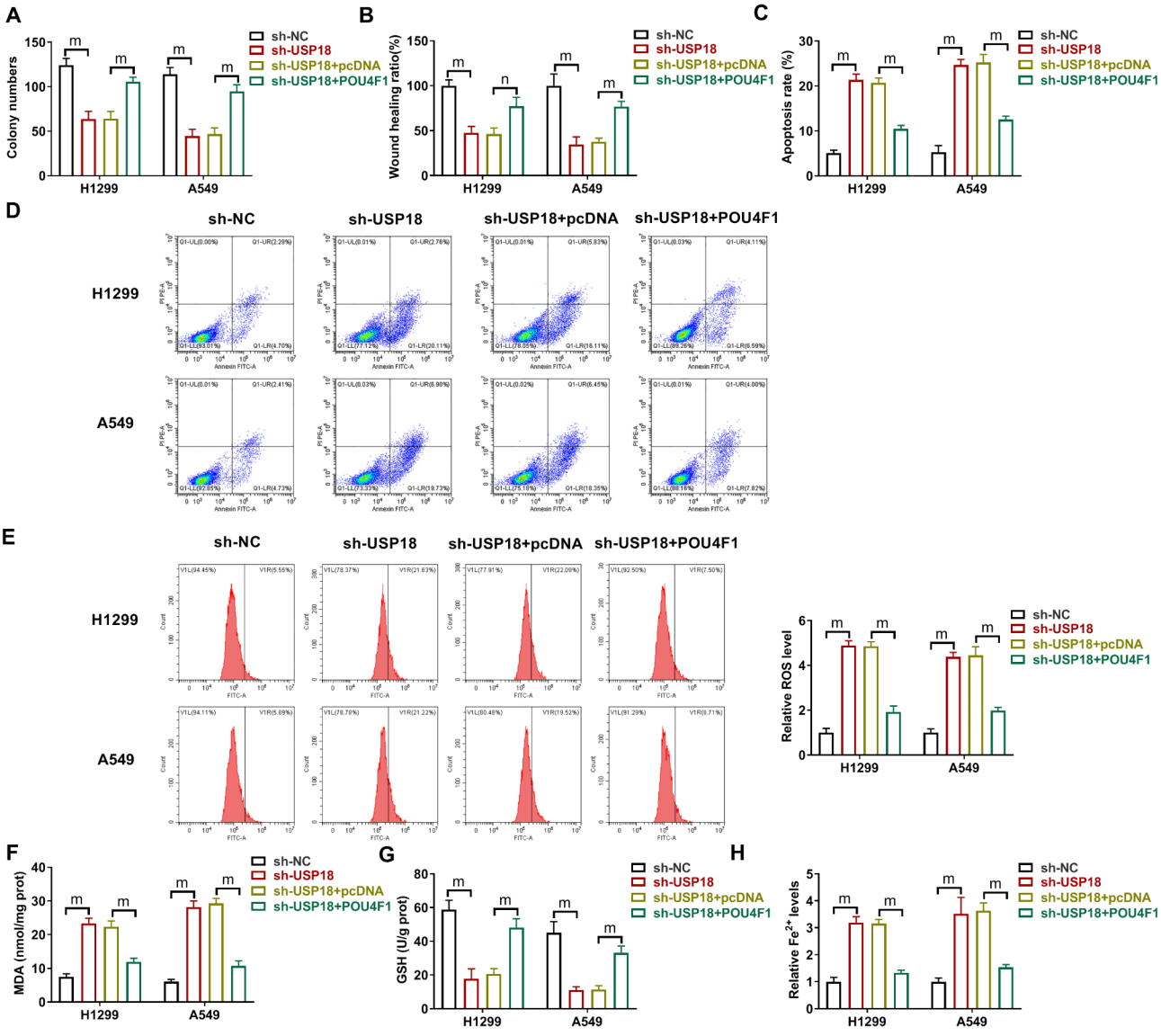

Fig. 4 Anti-tumor effects of USP18 silence were rescued by overexpression of POU4F1 in LUAD cells. Four groups: sh-NC, sh-USP18, sh-USP18 + pcDNA, sh-USP18 + POU4F1 were set in H1299 and A549 cells. **(A-D)** Examination of proliferation **(A)**, migration **(B)** and apoptosis **(C-D)** through colony formation assay, wound healing assay and flow cytometric assay. **(E-G)** ROS **(E)**, MDA **(F)**, and GSH **(G)** levels were measured via kits. **(H)** Fe^{2+} level detection using iron level assay. $^{n}P < 0.01$, $^{m}P < 0.001$

then this regulation was abated by PRKAA2 overexpression, which implied the direct and positive regulation of POU4F1 on PRKAA2 (Fig. 5K). The reversal of PRKAA2 protein expression was also found by POU4F1 transfection for sh-USP18, elucidating that USP18 regulated PRKAA2 via targeting POU4F1 (Fig. 5L). All in all, POU4F1 could promote PRKAA2 transcription and USP18 upregulated PRKAA2 expression via the deubiquitination of POU4F1.

PRKAA2 overexpression reversed the effects of POU4F1 knockdown on LUAD cells

Cell proliferation (Fig. 6A + Supplementary Fig. 2C) and migration (Fig. 6B + Supplementary Fig. 2D) were suppressed by silence of POU4F1, whereas these effects were balanced out after upregulation of PRKAA2. The promoting regulation of sh-POU4F1 in cell apoptosis (Fig. 6C-D), ROS level (Fig. 6E) and MDA level (Fig. 6F) was rescued after PRKAA2 was overexpressed. Knockdown of POU4F1 resulted in reduction of GSH level (Fig. 6G) and increase of Fe^{2+} level (Fig. 6H), which was offset after co-transfection with PRKAA2 in LUAD cells.

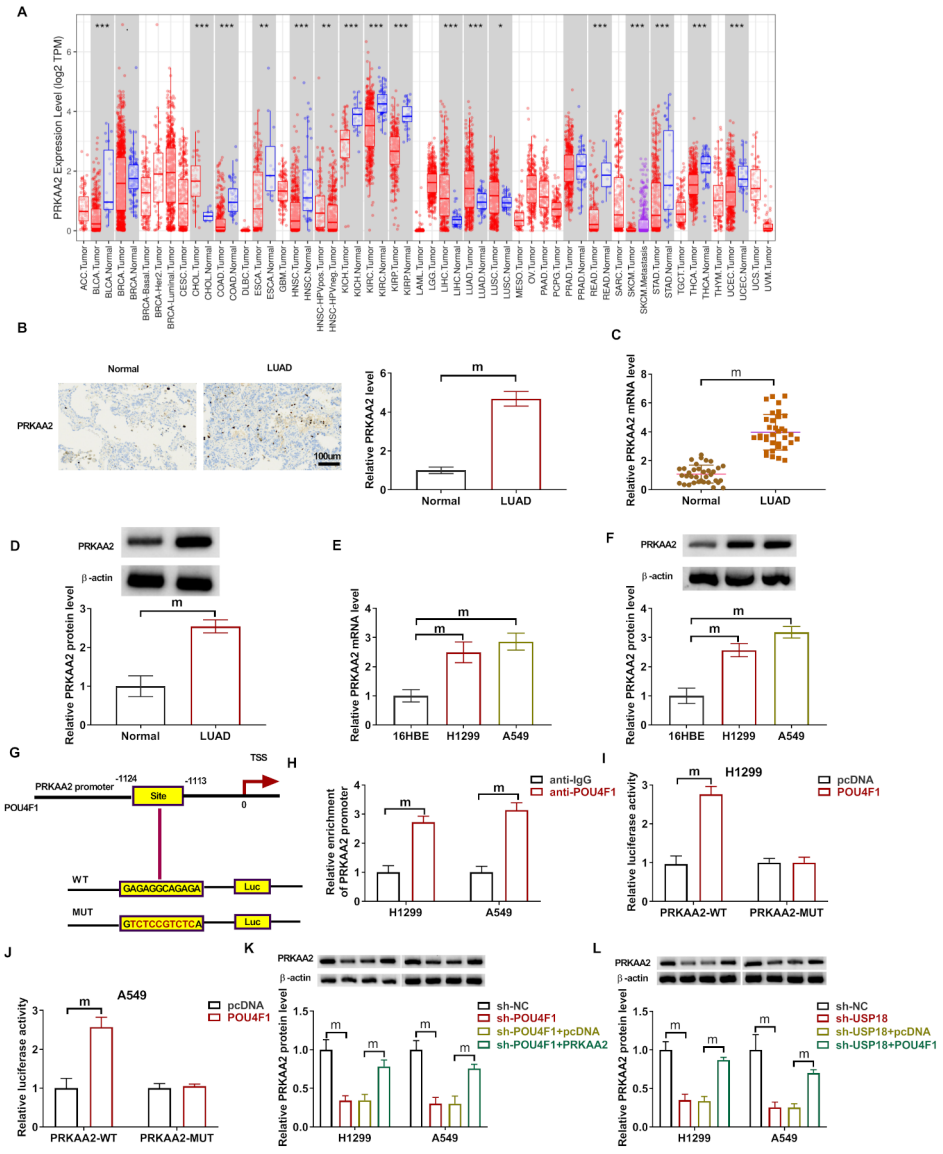

Fig. 5 USP18 affected PRKAA2 protein expression via deubiquitinating POU4F1 in LUAD cells. **(A)** PRKAA2 expression prediction in LUAD by Timer dataset. **(B)** IHC for protein examination of PRKAA2 in LUAD samples. According to the positive cells (brown), IHC quantification analysis was implemented by normalization of normal group to 1. **(C-D)** RT-qPCR **(C)** and western blot **(D)** were performed for expression analysis of PRKAA2 in LUAD tissues. **(E-F)** RT-qPCR **(E)** and western blot **(F)** were employed to measure mRNA and protein levels of PRKAA2 in LUAD cells. **(G)** Binding site between POU4F1 and PRKAA2 promoter in Jaspar dataset. **(H-J)** ChIP **(H)** and dual-luciferase reporter assay **(I-J)** for interaction analysis between POU4F1 and PRKAA2. **(K)** PRKAA2 protein analysis by western blot in sh-POU4F1, sh-POU4F1 + PRKAA2 and control groups. **(L)** Western blot was utilized for protein detection of PRKAA2 in sh-USP18, sh-USP18 + POU4F1 and control groups. $^{m}P < 0.001$

POU4F1 was involved in the regulation of LUAD cell progression via affecting PRKAA2 expression.

Silencing USP18 reduced tumor growth in vivo by mediating POU4F1/PRKAA2 axis

Xenograft model of sh-NC, sh-USP18, sh-USP18 + POU4F1, sh-POU4F1, or sh-POU4F1 + PRKAA2 group was established in nude mice with $n = 5$ in each group, as shown in Fig. 7A. Tumor volume and weight were decreased in sh-USP18 group or sh-POU4F1 group of xenograft mice relative to sh-NC group, while overexpression of POU4F1 or PRKAA2 respectively rescued the tumor growth inhibition caused by sh-USP18 or sh-POU4F1 (Fig. 7B-C). Western blot (Fig. 7D) and IHC (Fig. 7E) demonstrated that knockdown of USP18 or POU4F1 significantly downregulated the protein level of PRKAA2 in tumor tissues from mice, which was then offset following upregulation of POU4F1 or PRKAA2. The above results implicated that USP18 regulated

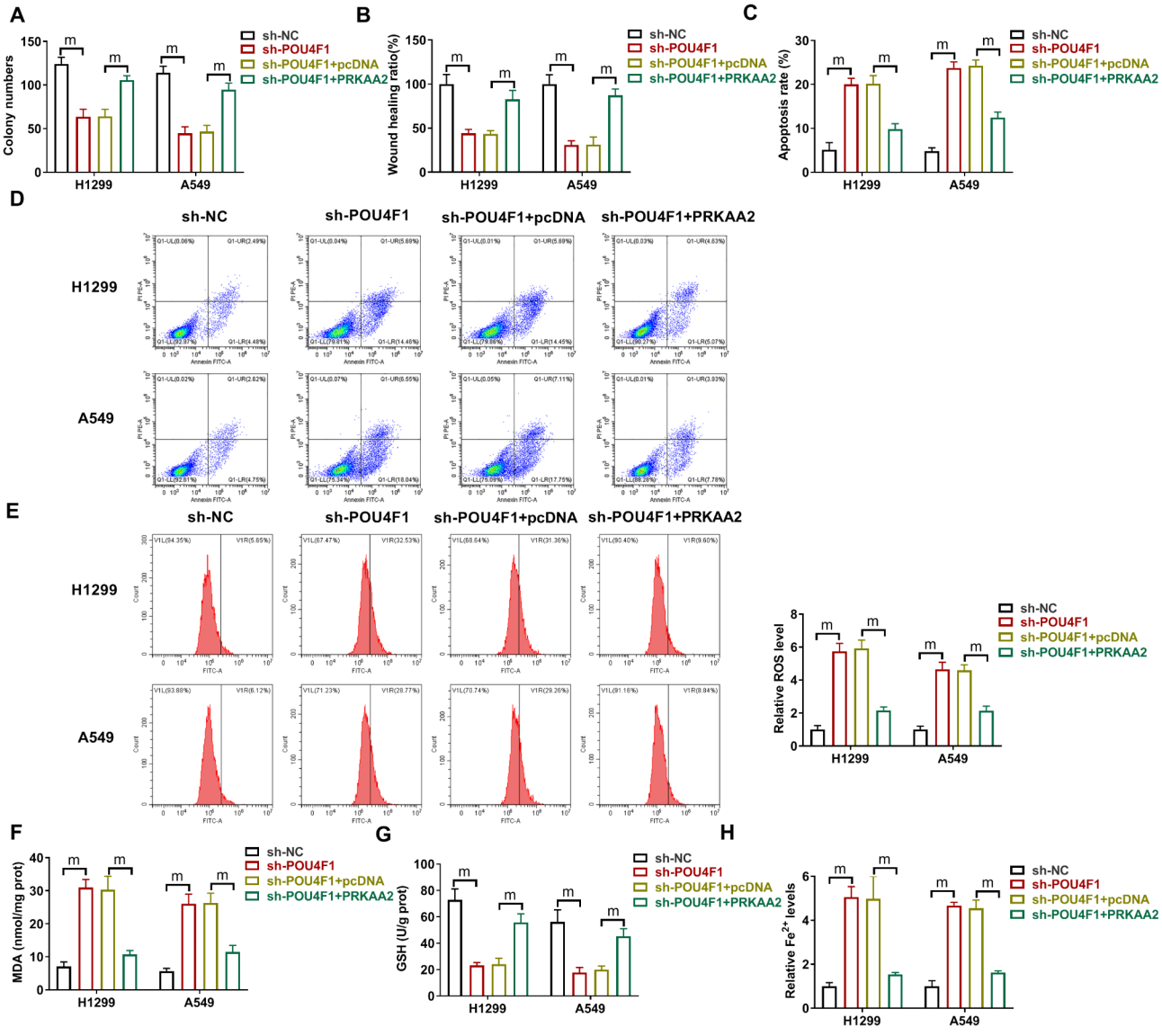

Fig. 6 PRKAA2 overexpression reversed the effects of POU4F1 knockdown on LUAD cells. Transfection of sh-NC, sh-POU4F1, sh-POU4F1 + pcDNA, sh-POU4F1 + PRKAA2 were performed in H1299 and A549 cells. **(A-D)** Colony formation assay, wound healing assay and flow cytometric assay for measuring proliferation **(A)**, migration **(B)** and apoptosis **(C-D)**. **(E-G)** ROS **(E)**, MDA **(F)**, and GSH **(G)** detection by kits. **(H)** Iron level assay for Fe^{2+} level examination. $^{m}P < 0.001$

tumor growth in LUAD via mediating POU4F1 to affect PRKAA2.

Discussion

DUBs can induce the post-translational modification "deubiquitination" to affect protein stability and function [22]. USPs are common DUBs to deubiquitinate target proteins, consequently involving in the regulation of cancer occurrence and development [23]. In this study, USP18 was confirmed to regulate various cellular processes of LUAD via stabilizing POU4F1 protein to enhance PRKAA2 expression.

Different USPs have been found to participate in lung cancer progression. For instance, USP15 and USP4 enhanced lung cancer cell proliferation ability [24], and USP14 functioned as a promising target in response to radiotherapy [25]. Also, USP18 was identified to play a vital role in various genres of tumors. Liu et al.. stated that ovarian cancer cell growth and motility were facilitated by USP18 [26]. Tan et al.. reported that USP18 promoted cell cycle progression and colony formation of breast cancer in vitro. Diao et al.. showed that USP18 contributed to proliferation and reduced apoptosis in cervical cancer [27]. The current expression analysis suggested that USP18 was an upregulated gene, consistent

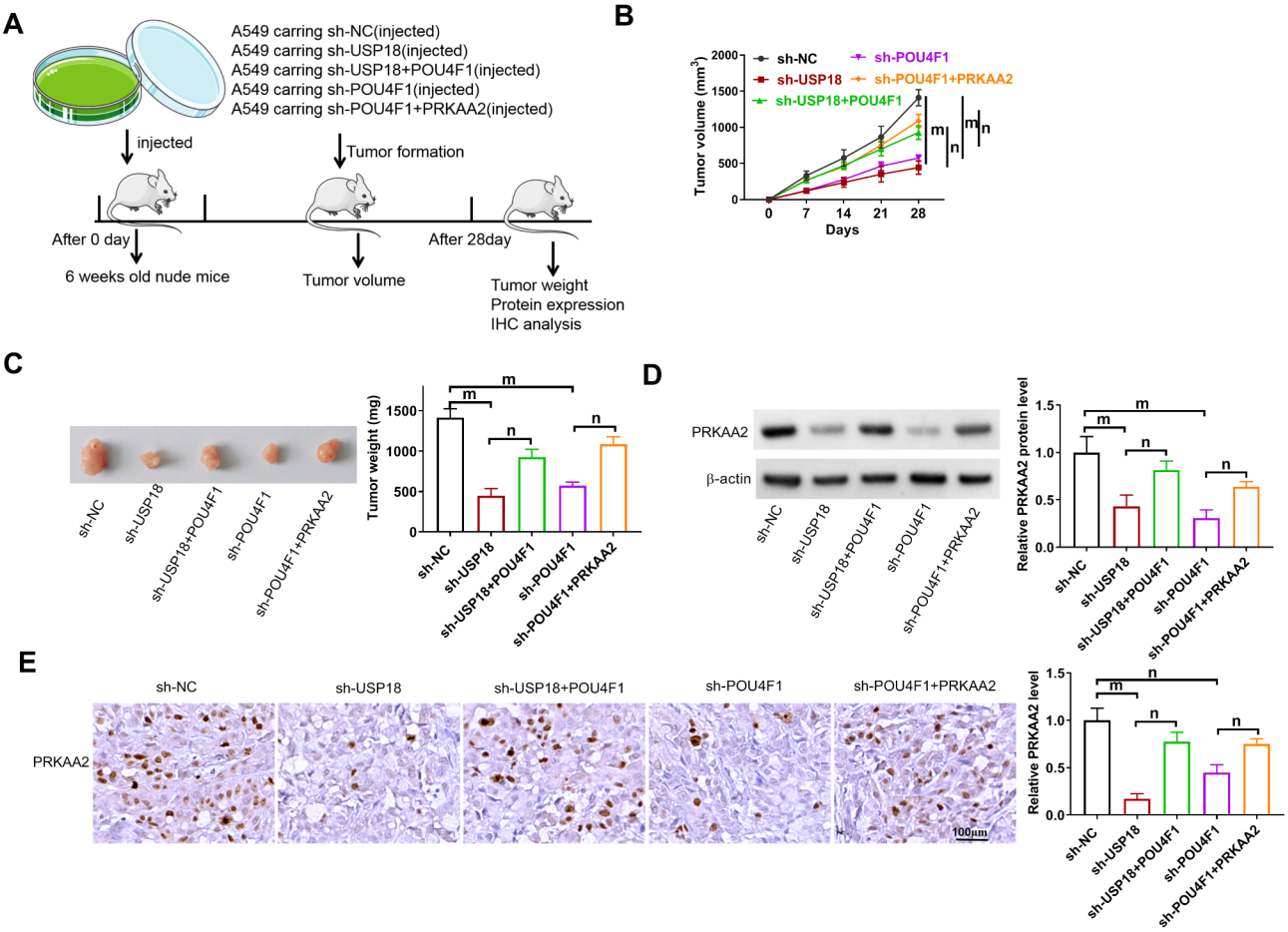

Fig. 7 Silencing USP18 reduced tumor growth in vivo by mediating POU4F1/PRKAA2 axis. **(A)** Establishment of xenograft model. **(B-C)** Tumor volume and weight of sh-NC, sh-USP18, sh-USP18 + POU4F1, sh-POU4F1, or sh-POU4F1 + PRKAA2 group. **(D)** Western blot was used to measure protein expression of PRKAA2 in tumor tissues. **(E)** PRKAA2 protein detection was carried out using IHC in tumor tissues. According to the positive cells (brown), IHC quantification analysis was completed by normalization of sh-NC group to 1. $^{n}P < 0.01$, $^{m}P < 0.001$

with the prediction of datasets. Functional assays displayed that USP18 downregulation suppressed cell proliferation and migration, suggesting that USP18 served as an oncogene in LUAD. DUBs exhibit the crucial regulation in cell death including apoptosis and ferroptosis [15]. Herein, we found cell apoptosis was enhanced as the result of USP18 knockdown. Ferroptosis is characterized by the superabundant accumulation of iron-dependent lipid peroxidation [28]. Our detection for oxidative indicators and Fe^{2+} showed that silencing USP18 accelerated oxidative stress and intracellular iron level, which demonstrated the anti-ferroptosis effect of USP18 in LUAD. Meanwhile, USP18 could enhance tumor growth in vivo.

DUBs can control protein function and degradation by removing ubiquitination of target proteins [29]. USP3 was manifested to promote lung cancer growth through upregulating RBM4 via acting as a DUB [30], and USP5 contributed to cell proliferation via stabilizing cyclin D1 protein level in lung cancer [31]. USP10 mediated ZEB1 ubiquitination and protein stability to repress colorectal cancer metastasis [32]. Our online prediction indicated POU4F1 as a substrate of USP18. Furthermore, we found that USP18 knockdown elevated the protein ubiquitination level of POU4F1 and reduced POU4F1 protein level, which implicated that USP18 promoted POU4F1 protein expression via inducing de-ubiquitination. Moreover, the functional influences of USP18 knockdown on LUAD cells were achieved by downregulating POU4F1. Thus, USP18 facilitated LUAD cell progression via stabilizing POU4F1 protein expression.

PRKAA2 is an AMP-activated protein kinase with important regulation in tumor growth and brain metastasis of melanoma [33]. Herein, PRKAA2 was found to be upregulated in LUAD. In addition, POU4F1 could promote transcription of PRKAA2 and the function of POU4F1 was related to the positive regulation for PRKAA2. Cheng et al.. suggested that USP53 regulate cell viability and metabolism of esophageal carcinoma via mediating AMPK pathway [34]. Our level analysis

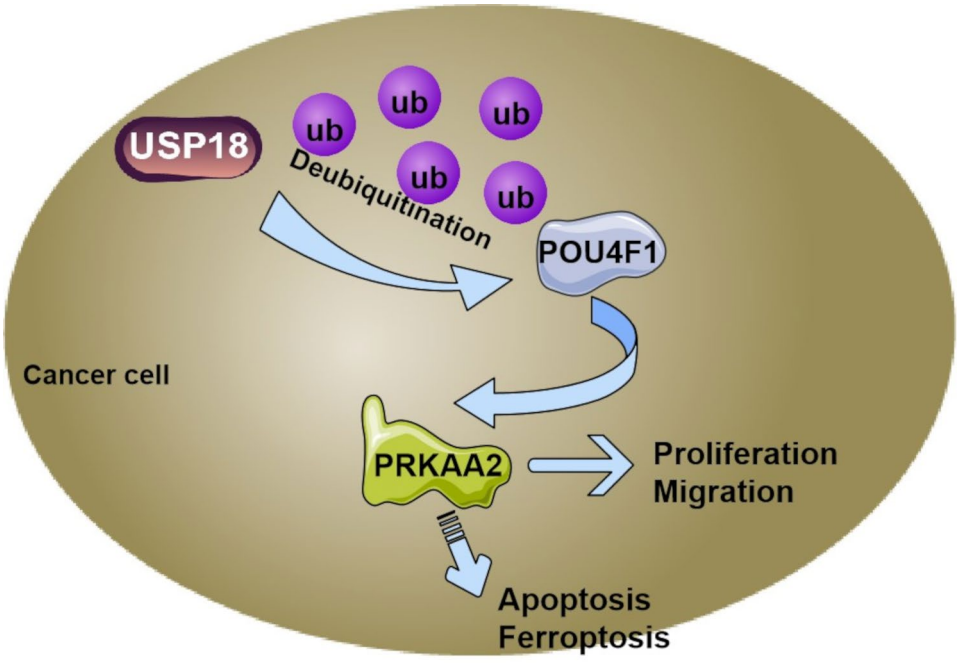

Fig. 8 USP18 regulated proliferation, migration, apoptosis, and ferroptosis by mediating deubiquitination of POU4F1 to affect PRKAA2

demonstrated that USP18 promoted PRKAA2 expression by enhancing de-ubiquitination of POU4F1.

However, there are some limitations in the current study. Firstly, the ubiquitination site of POU4F1 has not been ensured, and we will study the ub-site in the future. Secondly, it is unknown whether USP18 can affect LUAD progression by POU4F1/PRKAA2 axis through some signaling pathways. PRKAA2 downregulation has been revealed to activate the mTOR signaling [35]. The downstream pathways of USP18/POU4F1/PRKAA2 axis may be used as a future research highlight. In addition, USP18 could regulate ferroptosis via deubiquitinating SLC7A11 [36]. The regulatory potential of USP18 for SLC7A11 in LUAD is unexplored, and we may investigate this potential in the future. Nevertheless, our results still provide a novel understanding underlying migration and ferroptosis of LUAD.

In conclusion, USP18 regulated proliferation, migration, apoptosis and ferroptosis in LUAD through mediating deubiquitination of POU4F1 to affect PRKAA2 expression (Fig. 8). For the first time, USP18 was discovered to target POU4F1/PRKAA2 axis to function as an oncogene in LUAD. USP18 might be applied as a therapeutic target for LUAD.

Supplementary Information

The online version contains supplementary material available at https://doi.org/10.1186/s12885-025-13869-8.

Supplementary Material 1

Supplementary Material 2: fig. 1. Identification of the de-ubiquitination regulation of USP18 on POU4F1 (A-B) USP18 and POU4F1 protein levels were determined in H1299 and A549 cells treated with sh-NC, sh-USP18, sh-USP18 + MG132. (C) Co-IP detection for ubiquitination level of POU4F1 after treatment with pcDNA, USP18, or enzyme-dead USP18-C64S mutant. (D) Half-life of POU4F1 was detected by western blot in H1299 and A549 cells with CHX treatment under transfection of USP18 or USP18-C64S. $^{m}P < 0.001$.

Supplementary Material 3: fig. 2. The representative images of the colony formation and wound healing assays of figs. 4 and 6 (A-B) Images of colony formation (A) and wound healing (B) for Fig. 4A-B. (C-D) Images of colony formation (C) and wound healing (D) for Fig. 6A-B.

Supplementary Material 4

Acknowledgements

None.

Author contributions

Hui Deng designed and performed the research; Xinping Pan and Hui Deng analyzed the data; Xinping Pan wrote the manuscript. All authors read and approved the final manuscript.

Funding

None.

Data availability

The datasets used and/or analysed during the current study are available from the corresponding author on reasonable request.

Declarations

Ethics approval and consent to participate

Written informed consents were obtained from all participants and this study was approved by the Ethics Committee of Dingxi People's Hospital. All operations on mice have obtained ratification and approved by the Animal Ethical Committee of Dingxi People's Hospital.

Consent for publication

Not Applicable.

Competing interests
The authors declare that they have no conflicts of interest.

Received: 21 August 2024 / Accepted: 5 March 2025
Published online: 23 March 2025

References

1. Testa U, Pelosi E, Castelli G. Molecular charcterization of lung adenocarcinoma combining whole exome sequencing, copy number analysis and gene expression profiling. Expert Rev Mol Diagn. 2022;22(1):77–100.
2. Liu J, Zhang F, Wang J, Wang Y. MicroRNA–mediated regulation in lung adenocarcinoma: signaling pathways and potential therapeutic implications (Review). Oncol Rep 2023, 50(6).
3. Zhihua G, Jinghao L, Xin Z, Qing A, Zixian X, Haonan Z, Fayuan W, Zhaofeng T, Weiqiang Y, Linghua J. PCM1: A potential prognostic biomarker correlated with immune infiltration in lung adenocarcinoma. Curr Proteomics. 2023;20(3):208–21.
4. Wei X, Li X, Hu S, Cheng J, Cai R. Regulation of Ferroptosis in Lung Adenocarcinoma. *International journal of molecular sciences* 2023, 24(19).
5. Lin Y, Jiang S, Su J, Xie W, Rahmati M, Wu Y, Yang S, Ru Q, Li Y, Deng Z. Novel insights into the role of ubiquitination in osteoarthritis. Int Immunopharmacol. 2024;132:112026.
6. Ren J, Yu P, Liu S, Li R, Niu X, Chen Y, Zhang Z, Zhou F, Zhang L. Deubiquitylating enzymes in Cancer and immunity. Adv Sci. 2023;10(36):e2303807.
7. Zhou XJ, Li R, Liu X, Qu YQ. Advances in deubiquitinating enzymes in lung adenocarcinoma. J Cancer. 2021;12(18):5573–82.
8. Chen G, Du D, Wang H, Li H. The deubiquitinase USP22-Stabilized COL17A1 promotes lung adenocarcinoma progression. Clin Respir J. 2024;18(8):e13824.
9. Zhang T, Su F, Wang B, Liu L, Lu Y, Su H, Ling R, Yue P, Dai H, Yang T, et al. Ubiquitin specific peptidase 38 epigenetically regulates KLF transcription factor 5 to augment malignant progression of lung adenocarcinoma. Oncogene. 2024;43(16):1190–202.
10. Tecalco-Cruz AC, Ramirez-Jarquin JO, Zepeda-Cervantes J, Solleiro-Villavicencio H, Abraham-Juarez MJ. Ubiquitin-Specific Peptidase 18: A Multifaceted Protein Participating in Breast Cancer. In: *Breast Cancer.* edn. Edited by Mayrovitz HN. Brisbane (AU); 2022.
11. Dziamalek-Macioszczyk P, Harazna J, Stompor T. Versatility of USP18 in physiology and pathophysiology. Acta Biochim Pol. 2019;66(4):389–92.
12. Cai X, Feng S, Zhang J, Qiu W, Qian M, Wang Y. USP18 deubiquitinates and stabilizes Twist1 to promote epithelial-mesenchymal transition in glioblastoma cells. Am J Cancer Res. 2020;10(4):1156–69.
13. Liu X, Lu Y, Chen Z, Liu X, Hu W, Zheng L, Chen Y, Kurie JM, Shi M, Mustachio LM, et al. The Ubiquitin-Specific peptidase USP18 promotes lipolysis, fatty acid oxidation, and lung Cancer growth. Mol Cancer Res. 2021;19(4):667–77.
14. Chen Z, Zheng L, Chen Y, Liu X, Kawakami M, Mustachio LM, Roszik J, Ferry-Galow KV, Parchment RE, Liu X, et al. Loss of ubiquitin-specific peptidase 18 destabilizes 14-3-3zeta protein and represses lung cancer metastasis. Cancer Biol Ther. 2022;23(1):265–80.
15. Zhou Z, Song X, Kang R, Tang D. The emerging role of deubiquitinases in cell death. Biomolecules 2022, 12(12).
16. Zhang J, Miao N, Lao L, Deng W, Wang J, Zhu X, Huang Y, Lin H, Zeng W, Zhang W, et al. Activation of bivalent gene POU4F1 promotes and maintains Basal-like breast Cancer. Adv Sci. 2024;11(20):e2307660.
17. Li N, Chen S, Wang X, Zhang B, Zeng B, Sun C, Zheng K, Chen Q, Wang S. Identification of POU4F1 as a novel prognostic biomarker and therapeutic target in esophageal squamous cell carcinoma. Cancer Cell Int. 2024;24(1):280.
18. Ishii J, Sato H, Yazawa T, Shishido-Hara Y, Hiramatsu C, Nakatani Y, Kamma H. Class III/IV POU transcription factors expressed in small cell lung cancer cells are involved in proneural/neuroendocrine differentiation. Pathol Int. 2014;64(9):415–22.
19. Liu L, Guan X, Zhao Y, Wang X, Yin C, Liu Q, Li H. [Mechanism of miR-186-5p regulating PRKAA2 to promote ferroptosis in lung adenocarcinoma cells]. Zhongguo Fei Ai Za Zhi = Chin J Lung cancer. 2023;26(11):813–21.
20. Yuan LX, Luo M, Liu RY, Wang HX, Ju LL, Wang F, Cao YL, Wang ZC, Chen L. Hsa_circ_0005397 promotes hepatocellular carcinoma progression through EIF4A3. BMC Cancer. 2024;24(1):239.
21. Khokhlova ON, Borozdina NA, Sadovnikova ES, Pakhomova IA, Rudenko PA, Korolkova YV, Kozlov SA, Dyachenko IA. Comparative study of the aftereffect of CO(2) inhalation or Tiletamine-Zolazepam-Xylazine anesthesia on laboratory outbred rats and mice. Biomedicines 2022, 10(2).
22. Li KQ, Bai X, Ke AT, Ding SQ, Zhang CD, Dai DQ. Ubiquitin-specific proteases: from biological functions to potential therapeutic applications in gastric cancer. Biomed Pharmacother. 2024;173:116323.
23. Gao H, Yin J, Ji C, Yu X, Xue J, Guan X, Zhang S, Liu X, Xing F. Targeting ubiquitin specific proteases (USPs) in cancer immunotherapy: from basic research to preclinical application. J Exp Clin Cancer Res. 2023;42(1):225.
24. Das T, Lee EY, You HJ, Kim EE, Song EJ. USP15 and USP4 facilitate lung cancer cell proliferation by regulating the alternative splicing of SRSF1. Cell Death Discov. 2022;8(1):24.
25. Sharma A, Almasan A. USP14 regulates DNA damage response and is a target for radiosensitization in Non-Small cell lung Cancer. Int J Mol Sci 2020, 21(17).
26. Liu M, Gao S, Xu X, Zhang L, Xu B, Lu D. USP18 contributes to the proliferation and migration of ovarian cancer cells by regulating the AKT/mTOR signaling pathway. Acta Biochim Pol. 2022;69(2):417–22.
27. Diao W, Guo Q, Zhu C, Song Y, Feng H, Cao Y, Du M, Chen H. USP18 promotes cell proliferation and suppressed apoptosis in cervical cancer cells via activating AKT signaling pathway. BMC Cancer. 2020;20(1):741.
28. Li Q, Song Q, Pei H, Chen Y. Emerging mechanisms of ferroptosis and its implications in lung cancer. Chin Med J (Engl). 2024;137(7):818–29.
29. Ye B, Zhou H, Chen Y, Luo W, Lin W, Zhao Y, Han J, Han X, Huang W, Wu G, et al. USP25 ameliorates pathological cardiac hypertrophy by stabilizing SERCA2a in cardiomyocytes. Circ Res. 2023;132(4):465–80.
30. Liao XH, Wang Y, Zhong B, Zhu SY. USP3 promotes proliferation of non-small cell lung cancer through regulating RBM4. Eur Rev Med Pharmacol Sci. 2020;24(6):3143–51.
31. Zhang Z, Cui Z, Xie Z, Li C, Xu C, Guo X, Yu J, Chen T, Facchinetti F, Bohnenberger H, et al. Deubiquitinase USP5 promotes non-small cell lung cancer cell proliferation by stabilizing Cyclin D1. Transl Lung Cancer Res. 2021;10(10):3995–4011.
32. Sun L, Yu J, Guinney J, Qin B, Sinicrope FA. USP10 regulates ZEB1 ubiquitination and protein stability to inhibit ZEB1-Mediated colorectal Cancer metastasis. Mol Cancer Res. 2023;21(6):578–90.
33. Yuan P, Teng D, de Groot E, Li M, Trousil S, Shen CH, Roszik J, Davies MA, Gopal YNV, Zheng B. Loss of AMPKalpha2 promotes melanoma tumor growth and brain metastasis. iScience. 2023;26(6):106791.
34. Cheng W, Tang Y, Tong X, Zhou Q, Xie J, Wang J, Han Y, Ta N, Ye Z. USP53 activated by H3K27 acetylation regulates cell viability, apoptosis and metabolism in esophageal carcinoma via the AMPK signaling pathway. Carcinogenesis. 2022;43(4):349–59.
35. Zhuang J, Shen L, Li M, Sun J, Hao J, Li J, Zhu Z, Ge S, Zhang D, Guo H, et al. Cancer-Associated Fibroblast-Derived miR-146a-5p generates a niche that promotes bladder Cancer stemness and chemoresistance. Cancer Res. 2023;83(10):1611–27.
36. Zhou Q, Yu H, Chen Y, Ren J, Lu Y, Sun Y. The CRL3(KCTD10) ubiquitin ligase-USP18 axis coordinately regulates cystine uptake and ferroptosis by modulating SLC7A11. Proc Natl Acad Sci USA. 2024;121(28):e2320655121.

Publisher's note