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Original Article

Cezanne contributes to cancer progression by playing a key role in the deubiquitination of IGF-1R

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Abstract: *Background:* Degradation of insulin-like growth factor 1 receptor (IGF-1R) is mediated by internalization and endocytosis, for which ubiquitin-proteasome pathways play as a regulatory system. Cezanne expression is positively associated with IGF-1R expression. High Cezanne expression correlates with poor patient survival in NSCLC, yet the underlying mechanisms are not well defined. *Methods:* Co-Immunoprecipitation assay was performed to investigate the interactions between Cezanne and IGF-1R. A xenograft model was established to assess the efficacy of Cezanne on cancer progression in vivo. Cezanne overexpressing and Cezanne knockdown NSCLC cell lines were generated using lentiviral vectors. The effects of Cezanne and IGF-1R on cell proliferation of non-small-cell lung cancer were evaluated via Sulforhodamine B assay and colony formation assays. *Results:* Here, through co-Immunoprecipitation assay, we find Cezanne interacts with IGF-1R in tumor cells. Depletion of Cezanne promotes the ubiquitination and degradation of IGF-1R. Congruently, Cezanne regulates the protein level of IGF-1R and downstream AKT signaling pathway. Cezanne promotes proliferation of tumor cells in vitro and in vivo. In line with the change of IGF-1R downstream signaling pathway, IGF-1-induced growth signals recover cell proliferation of tumor cells with Cezanne knockdown. Conclusion: Mechanistically, Cezanne directly targets IGF-1R by deubiquitination and stabilization. This leads to AKT activation, which bolsters tumor cell growth in vitro and in vivo. These findings reveal Cezanne as a regulator of tumor cell proliferation via IGF-1R signaling pathway and a potential target for NSCLC therapy.

Keywords: IGF-1R, Cezanne, deubiquitination, NSCLC

Introduction

Insulin-like growth factor 1 receptor (IGF-1R) has been recognized as a receptor tyrosine kinase (RTK) with crucial roles in cancer biology in recent two decades [1]. IGF-1R is a heterotetrameric receptor tyrosine kinase which is composed of two extracellular α-subunits involved in ligand binding and two transmembrane β-subunits composing a transmembrane domain, an intracellular tyrosine kinase domain and a C-terminal domain [2]. Once combined with ligands, the intracellular kinase will be activated through an autophosphorylation-induced conformational change and so will the downstream signaling cascades, mainly including two major downstream signaling pathways, the PI3K-Akt pathway and the Shc-Ras-MAPK pathway [3]. And ultimately it leads to major biological outcomes such as tumor transformation, maintenance of tumorigenicity, promotion of cell growth, and prevention of apoptosis. Generally, active receptors must be cleared from cell surface to desensitize cells to mitogenic signals. Endocytosis, a process by which eukaryotic cells internalize plasma membrane (PM), along with cell-surface receptors and diverse soluble molecules, plays an important role in the termination of the signal, which are dependent on receptor ubiquitination [4-8].

Ubiquitination, an covalent attachment of ubiquitin to a substrate catalyzed by the E1, E2 and E3 three-enzyme cascade [9], which links the C

terminus of ubiquitin via an isopeptide bond mostly to ε-amino group of a lysine of the substrate [10], is an important regulation mechanism for the control of various biological processes, including cell-cycle progression, DNA damage repair, signal transduction, transcriptional regulation, and regulation of the localization, stability, and activity of receptor tyrosine kinases [11-13]. Ubiquitination is implicated in receptor tyrosine kinase internalization and endocytosis [14]. And thereby comes the termination of signal and degradation of receptors.

Recent studies have demonstrated that IGF-1R is a substrate for ubiquitination. E3 ligases, including Nedd4, Mdm2 and c-Cbl, have been revealed to mediate IGF-IR ubiquitination [15-17]. Upon phosphorylation, IGF-1R will be attached by ubiquitin molecules and then recognized by ubiquitin-binding adaptors harboring ubiquitin-binding domains (UBDs), and subsequently recruited to the plasma membrane thereby endocytosed [18].

Major posttranslational modifications including ubiquitination process are reversible just as every action provokes a reaction. While there is a complex ubiquitination system for ubiquitinating proteins and target them for modification or degradation, there also correspondingly exists a deubiquitination program [19]. It is the process of cleaving ubiquitin molecules from ubiquitin conjugated protein substrates by deubiquitinating enzymes (DUBs), which can rescue proteins from degradative pathways or lead to reversion of ubiquitin signaling [20-22]. Over one hundred genes potentially coding for DUBs are identified in human genome and can be classified into five different varieties including JAB1/MPN/Mov34 (JAMM), ubiquitin C-terminal hydrolase (UCHs), ubiquitin-specific protease (USPs), ovarian tumor domain (OTUDs) and Josephin. JAMM belongs to the superfamily of zinc-dependent metalloproteases, and other DUBs are classical papain-like cysteine proteases [20, 21].

Cezanne (OTUD7B), a member of OTUDs family, shows a preference for K11-uniquitin linkages [23]. Since Cezanne was reported as a novel type of deubiquitination enzyme in 2003, studies have been conducted to investigate its role of regulating different proteins in various cancers [24-27]. And Cezanne has been reported to regulate NF-κB signaling [25, 28], EGFR trafficking [26], APC/C substrates [29] and homeostasis of HIF-1α and HIF-2α [30, 31], yet the functions of Cezanne in the development of NSCLC remain largely unknown.

Our previous study showed that Cezanne predicted poor outcomes in lung adenocarcinoma, of which the expression was significantly associated with IGF-1R expression [32]. The aim of the present study is to investigate the relationship between Cezanne and IGF-1R, and the underlying mechanism of Cezanne contributing to cancer progression. Herein, our results provide evidence that Cezanne is involved in deubiquitination and degradation of the IGF-1R. Mechanistically, Cezanne directly targets IGF-1R by deubiquitination and stabilization, leading to AKT activation, which bolsters tumor cell growth in vitro and in vivo. Therefore, our study highlights a crucial regulator implicated IGF-1R expression, and suggests a potential therapeutic target for NSCLC intervention.

Materials and methods

Bioinformatics analysis

Survival analyses between Cezanne high-expression and low-expression groups were conducted with K-M survival curves which were drawn from Kaplan-Meier Plotter database (*http://kmplot.com/analysis/index.php?p=service&cancer=lung*).

Patient tissues

20 pair frozen tissues of patients with primary NSCLC who had undergone surgical resection in Shandong Provincial Hospital (Jinan, China) were collected for qRT-PCR. Tumor samples of 108 NSCLC patients with surgical resection were enrolled for Immunohistochemistry (IHC) staining. Overall survival (OS) referred to the time from date of surgery to death for any cause. If the patient was alive or out of touch, the endpoint of OS was the date of last follow-up. This study was approved by the Ethical Committee of Shandong Provincial Hospital, Cheeloo College of Medicine, Shandong University. All experiments were performed in accordance with relevant guidelines and regulations.

Quantitative real-time polymerase chain reaction (qRT-PCR)

Total RNA was extracted from NSCLC frozen tumor tissues, the corresponding peritumoral

normal tissues using RNAiso Plus (Takara, Dalian, China). The mRNA was converted into cDNA using PrimeScript™ RT reagent Kit with gDNA Eraser (Takara, Japan). Afterwards, cDNA was amplified with SYBR Premix Ex Taq (TliRNaseH Plus, Takara, Japan) and conditions for the PCR reactions were 10 min at 95°C followed by 40 cycles of 15 s at 95°C and 1 min at 60°C. Gene expression levels were assayed by qRT-PCR using the Roche LightCycler® 480 system. All assays were performed in triplicate and $2^{-\Delta\Delta Ct}$ method was used to obtain relative quantitation (RQ) values, with 18S rRNA as endogenous control.

The primers used for qRT-PCR are listed as followed: OTUD7B rRNA-1: 5'>GAGGATCACATGACCCTGGAC<3'; OTUD7B rRNA-2: 5'>CGGCATTCACATCCCAATTC<3'; 18S rRNA-1: 5'>AAACGGCTACCACATCCAAG<3'; 18S rRNA-2: 5'>CCTCCAATGGATCCTCGTTA<3'.

Immunohistochemistry (IHC) staining

56 lung adenocarcinoma (ADC) specimens and 52 squamous carcinoma (SCC) specimens were analyzed by IHC staining. After resection, samples were fixed by formalin, embedded by paraffin, cut 4 μm thick, mounted on glass slides, deparaffined at 60°C for 2 h, processed in turpentine, rehydrated via ordinal 100, 95, 85 and 75% alcohol, washed using phosphate-buffered saline (PBS), heated in EDTA antigen-repairing solution (0.01 M, pH 8.0) for 2 min. Afterward, we used 3% hydrogen peroxide to block endogenous peroxidase in samples. Non-specific staining was blocked with 10% normal goat serum. Then, tissue specimens were incubated with Cezanne (OTUD7B) mouse monoclonal antibody (Santa Cruz, USA: 1:100) overnight at 4°C. 3, 3'-diaminobenzidine (DAB) kit (Zhongshan Biotechnology, Beijing, China) was used for dyeing.

IHC scoring

All specimens were scored according to the intensity of dyed color and the percentage of positive cells stained. The intensity of staining was graded as: 0, no color; 1, light yellow; 2, light brown; 3, deep brown, and grades according to positive cell numbers were determined as: 0 (<5%), 1 (5~25%), 2 (26~50%), 3 (51~75%), 4 (>75%). We added two scores together to comprehensive evaluate the staining. Score 0~2 (low IHC score) were defined as low expression. Once the value of total number ≥3 (high IHC score), the expression was considered as high expression.

Cell culture

All cell lines were purchased from the American Type Culture Collection (ATCC, USA). HEK293T cells were cultured in DMEM (HyClone, USA), A549 cells were cultured in F12K (Macgene, USA), and the other cell lines were cultured in RIPM H1640 (HyClone, USA), supplemented with 10% fetal bovine serum (BI, Israel) in a humidified atmosphere of 5% CO_2 and 37°C according to protocol.

Transfections

Lentiviruses were produced using HEK293T cells. Briefly 6×10^6 cells were plated in 10 cm plates and simultaneously transfected with 5 μg Cezanne shRNA plasmid or Cezanne overexpression plasmid (OBiO Technology, Shanghai, China), 5 μg of pSPAX2 (Addgene, USA) and 2 μg pMD2.G (Addgene, USA). EZ Trans (Life iLAB Biotech, Shanghai, China) was used for transfection according to manufacturer's instructions. Medium was changed after 24 hours and lentivirus containing medium was collected 48 hours post transfection. For lentiviral transduction in a 6-well plate, 600 μl regular medium, 400 μl viral supernatant and 6 μg/ml polybrene were used.

Co-immunoprecipitation (Co-IP)

Cells were lysed with IP lysis buffer [20 mM Tris (pH 7.5), 150 mM NaCl, 1% Triton X-100, with protease inhibitor tablet (Roche)] and then incubated with Dynabeads protein G (10 μL) (Invitrogen) and 1 μg antibody [OTUD7B or IGF-1Rβ (Santa Cruz, USA)] overnight at 4°C on a rotator platform. A magnetic holder was used to collect the immunoprecipitates, with the supernatant discarded and the beads washed three times with lysis buffer, and finally dissolved in the sample buffer for western blotting.

Western blot analysis

Protein samples were dissolved in lithium dodecyl sulfate (LDS) sample buffer (Invitrogen). Equivalent amounts of total protein extract were separated on 10% SDS-PAGE gels (90 V

for 30 min and 120 V for 60 min) and transferred to polyvinylidene fluoride membranes. The transfer was carried out at 100 V for 2 h using a Bio-Rad transfer apparatus. Membranes were then blocked for 1 h at room temperature in 5% BSA solution. Appropriate primary antibody was incubated overnight at 4°C. The primary antibodies were listed as followed: Cezanne (OTUD7B) (Proteintech, USA: 1:1000); Akt, p-Akt, p-IGF-1Rβ and p-Erk1/2 (T202/Y204) (Cell Signaling Technology, USA: 1:1000); GAPDH, JNK and p38α (Santa Cruz, USA: 1:1000); Erk1/2, p-p38 MAPK (T180), p-p38 MAPK (Y182), p-JNK/SAPK (Thr183/Tyr185) and IGF-1Rβ (Abgent, UK: 1:1000). After washing with TBST, the membrane was incubated with corresponding HRP-labeled secondary antibody (Santa Cruz, USA: 1:10000) for 1 h. The detection of part assays was performed with enhanced chemiluminescent substrate (Pierce) and exposed to X-ray film. The other immunoreactive bands were visualized using ECL kit and FluorChem E system (Proteinsimple, USA).

Proliferation assays

Cells were resuspended and seeded in 96-well plate with a density of 1500 cells per well. After 6 hours, the first plate was fixed with 10% cold trichloroacetic acid for at least 24 h. Then the other samples were collected in this way every 24 h. After all the samples were fixed, the plates were washed five times by submersion in tap water and excess water was removed. Then they were stained by Sulforhodamine B sodium salt (SRB) (Sigma, USA) for 30 min and washed three times by 1% (vol/vol) acetic acid. About 24 hours later, after the plates were dry, 150 ul 10 mmol/L Tris was added to each well. Then the absorbance was measured at 562 nm in a microplate reader (Thermo Fisher, USA), and the results were analyzed with GraphPad Prism 7.

Colony formation assay

Cells were seeded in 6-well plates at a density of 1000 cells per well and cultured at 37°C for 14 days. At the end of the incubation, the colonies were carefully washed with phosphate-buffered saline (PBS), then fixed with 4% paraformaldehyde for 30 min and stained with 0.1% crystal violet for 30 min. Images of the clone formation were obtained by scanning the cell culture dishes.

A549 tumor xenografts

BALA/c-nu mice was purchased from Beijing Huafukang Biotechnology Co., Ltd. (Beijing, China). All the animal care and experiments were approved by the Ethical Committee of Shandong Provincial Hospital Affiliated to Shandong University. The animal house was maintained at temperature of 22 ± 2°C with relative humidity of 50 ± 15% and 12 h dark/light cycle. To generate A549 tumor-bearing xenografts, A549 cells at a concentration of 2×10^6 mixed with 50% Matrigel (BD Biosciences) were implanted subcutaneously in the thighs of 6-week-old male nude mice. When the tumor volume reached approximately 100 mm^3, the anti-cancer activity of Cezanne knockdown in vivo was evaluated by monitoring each tumor volumes every 3 days by Vernier caliper and tumor volume was indicated by $a \times b^2/2$ (a for long diameter; b for short diameter). Mice were sacrificed at 40 days after injection with no tumor volume reaching 2000 mm^3.

Statistical analysis

Kaplan-Meier method was used to generate the survival curve, and the significance of difference was calculated by log-rank test. The two-sided *p* values <0.05 was considered to be statistically significant. All data were analyzed by GraphPad Prism 7.

Results

Expression and prognostic value of cezanne in NSCLC

As shown in **Figure 1A**, the Kaplan-Meier survival curve suggested that high Cezanne expression level was associated with poor prognosis in lung cancer (HR=1.27, 95% CI: 1.12-1.44, P<0.001), which was drawn using Kaplan-Meier Plotter database including 1926 lung cancer patients. Total RNA was extracted from 20 NSCLC frozen tissues along with tumor-adjacent lung tissues. The mRNA expression level of Cezanne was measured by qRT-PCR using 18S mRNA as internal standard. Of the 20 paired tissues, the mRNA expression level of Cezanne was at significantly higher level in 14 NSCLC tissues relative to adjacent

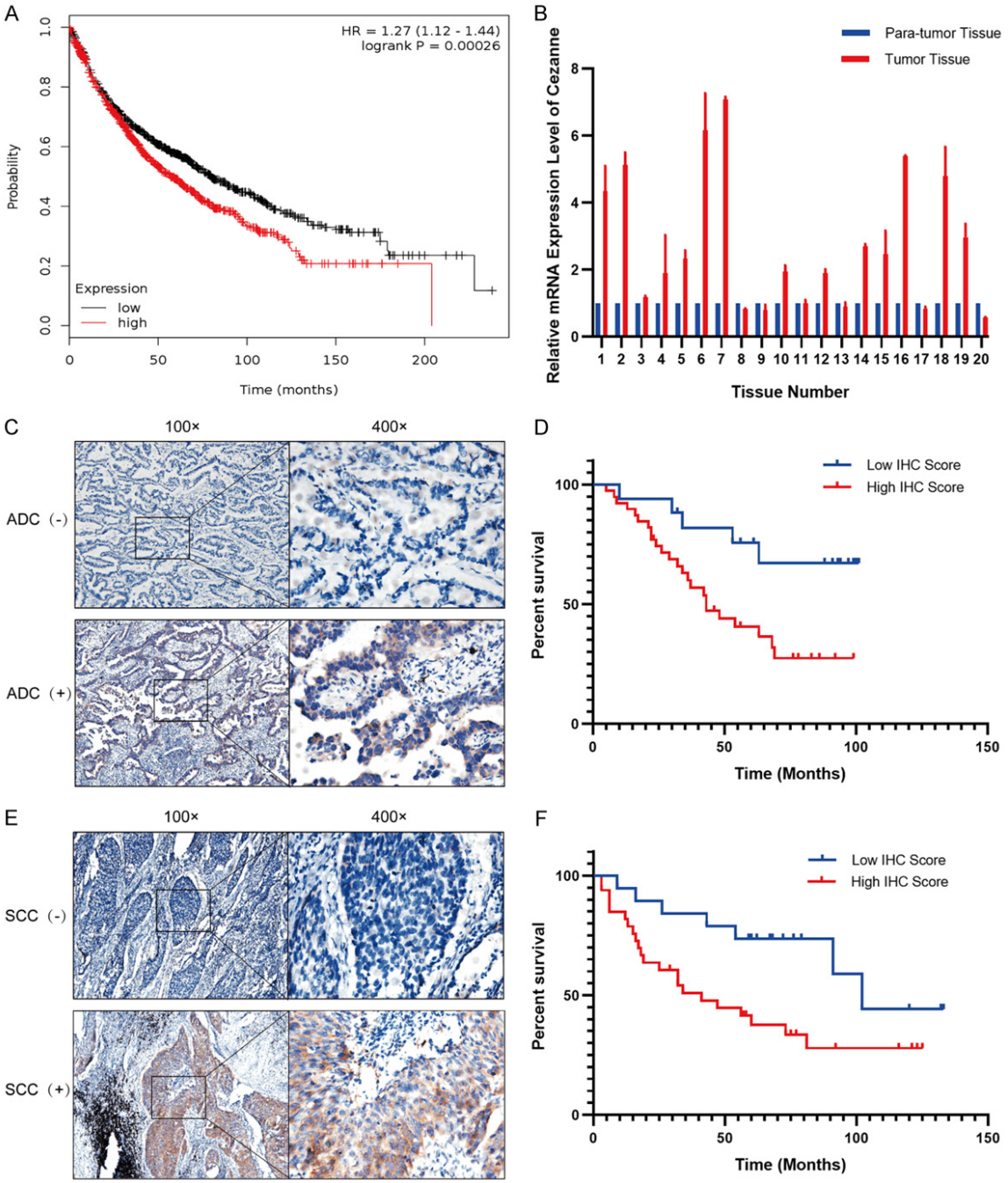

Figure 1. Expression and Prognostic value of Cezanne in NSCLC. A. Survival curve from Kaplan Meier-plotter database, HR=1.27 (1.12-1.44), P<0.001. Kaplan-Meier method was used to generate the survival curve, and the significance of difference was calculated by log-rank test. B. Expression of Cezanne in 20 paired NSCLC tissues compared with tumor-adjacent normal lung tissues measured by qRT-PCR. The difference of Cezanne expression was analyzed by pared t-test. C. Positive and negative expression of Cezanne in ADC tissues by IHC (100× & 400×). -, negative; +, positive. D. Kaplan Meier survival curve of Cezanne of the 56 ADC tissues; *P<0.05. Kaplan-Meier method was used to generate the survival curve, and the significance of difference was calculated by log-rank test. E. Positive and negative expression of Cezanne in SCC tissues by IHC (100× & 400×). -, negative; +, positive. F. Kaplan Meier survival curve of Cezanne of the 52 SCC tissues; *P<0.05. The significance of difference was calculated by log-rank test.

counterparts (**Figure 1B**). Coincidentally, the IHC staining of Cezanne in 56 ADC and 52 SCC tissues revealed that high Cezanne expression level predicted poor prognosis both in ADC and

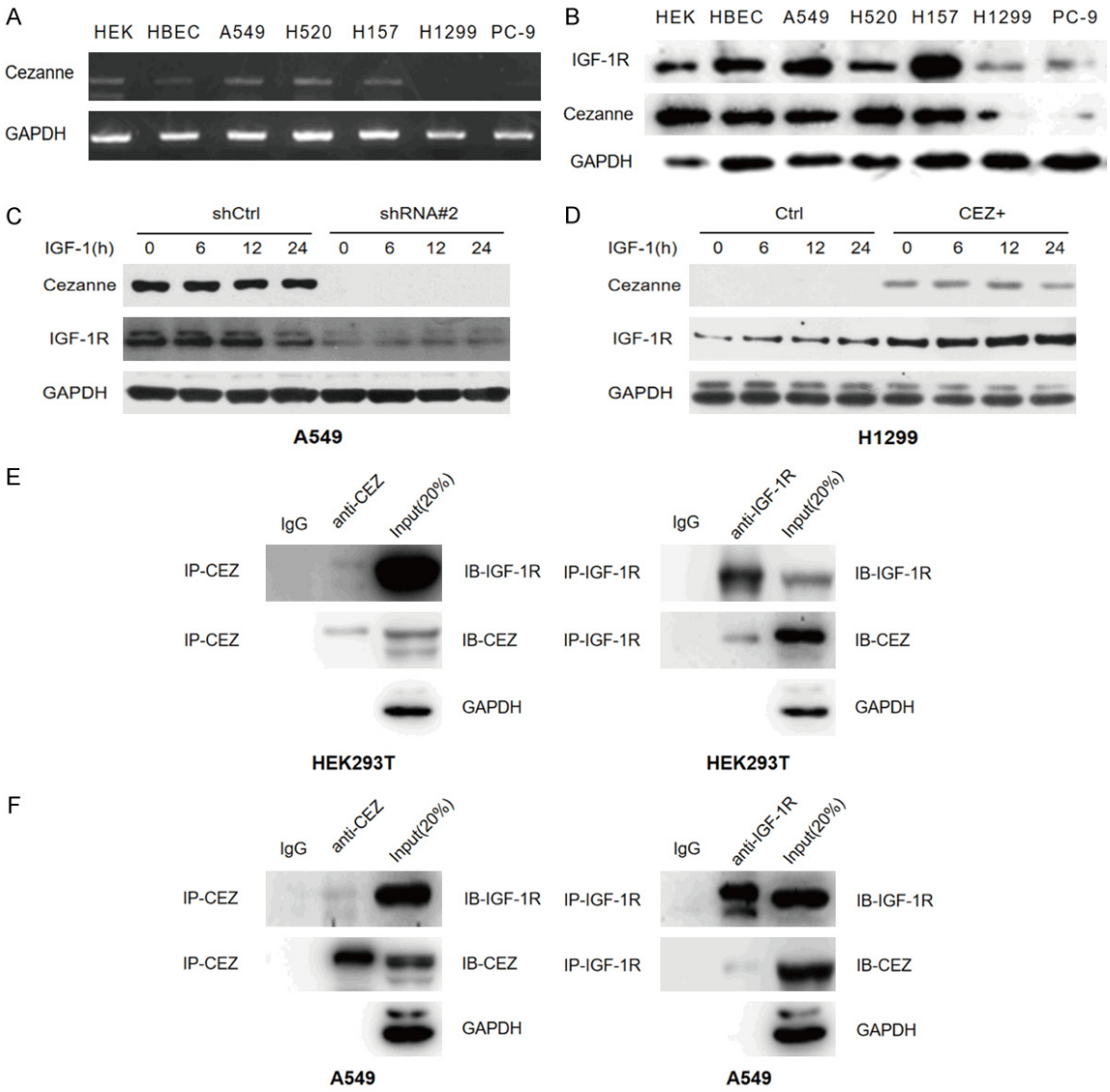

Figure 2. Cezanne regulated IGF-1R protein level and physically interacted with IGF-1R. A. Expression of Cezanne in different cell lines detected by PCR. B. Expression of Cezanne and IGF-1R in different cell lines detected by western blot. C. A549 cells were respectively transfected with shRNA and control plasmid, starved for 12 h, treated with IGF-1 for 0, 6, 12, 24 h. IGF-1R and Cezanne were detected via western blot. D. H1299 cells were respectively transfected with Cezanne plasmid and empty vector plasmid, starved for 12 h, treated with IGF-1 for 0, 6, 12, 24 h. IGF-1R, Cezanne, GAPDH were detected via western blot. E. Whole cell extracts of HEK293T cells were isolated for co-immunoprecipitation experiments. F. Whole cell extracts of A549 cells were isolated for co-immunoprecipitation experiments.

SCC ($P<0.05$; **Figure 1C-F**), suggesting the potential oncogenic activity of Cezanne in NSCLC.

Cezanne regulated IGF-1R protein levels

IGF-1R expression was positively associated with Cezanne expression. As shown in **Figure 2**, the expression level of Cezanne and IGF-1R was investigated further by PCR and western blot in HEK293T, HBEC and NSCLC cell lines including A549, H520, H157, H1299 and PC-9. Cezanne was expressed with a relatively higher level in A549 cells and with a relatively lower level in H1299 cells. Then, we seeded and transfected A549 cells with shRNA and corresponding control plasmid. And H1299 cells was transfected with Cezanne overexpression plasmid with empty vector as control. Cells were starved for 8 hours subsequently and stimulated with IGF-1 for 0, 6, 12, 24 hours,

respectively. Total IGF-1R, Cezanne and GAPDH were detected via western blot. The results demonstrated that IGF-1R was downregulated in A549 cells with Cezanne knockdown compared with control (**Figure 2C**). Consistently, overexpressing Cezanne displayed up-regulated IGR-1R in H1299 cells (**Figure 2D**).

Cezanne physically interacted with IGF-1R

We next checked if there was any interaction between Cezanne and IGF-1R. Whole cell extracts of HEK293T cells were isolated for detection by co-immunoprecipitation (co-IP) assays. Results confirmed the endogenous interaction between Cezanne and IGF-1R (**Figure 2E**). Similar results could also be seen in A549 cells (**Figure 2F**).

Cezanne stabilized and deubiquitinized IGF-1R

The canonical function of Cezanne was to prevent degradation of substrates via deubiquitination. Thus, we sought to determine whether Cezanne participated in regulating the stability of IGF-1R by deubiquitination. Degradation of IGF-1R was reported to be mediated by ubiquitination-proteasome pathway [12, 22]. The level of IGF-1R was downregulated in A549 cells treated with PR-619, an inhibitor of deubiquitinase, compared to blank control (**Figure 3A**). Further, we exogenously manipulated the level of Cezanne through its downregulation in A549 cells. On the one hand, when combined with cycloheximide (CHX) (10 μg/ml) treatment to block de novo protein biosynthesis, we found that Cezanne downregulation cells promoted the degradation of IGF-1R (**Figure 3B**). On the other hand, the degradation of both Cezanne and IGF-1R were inhibited when combined with MG132 (100 μg/ml) treatment to block the biological activity of proteasome (**Figure 3C**, **3D**).

Cezanne overexpression plasmid and empty vector plasmid were transfected into HEK293T cells. The results presented in **Figure 3F** and **3G** confirmed that Cezanne overexpression was associated with remarkably reduced ubiquitination of IGF-1R, which became more obvious when the expression of Cezanne increased. Moreover, the deubiquitination was regulated by IGF-1 stimulation. Notably, silencing Cezanne resulted in more ubiquitination of IGF-1R compared with Cezanne overexpression in A549 cells (**Figure 3E**).

Knockdown of Cezanne suppressed tumor cell proliferation in vitro and in vivo

High Cezanne expression level closely correlated with poor prognosis in NSCLC patients. We therefore tested biological effects of Cezanne on lung cancer cells both in vitro and in vivo. As shown in **Figure 4A-C**, A549 cell growth in vitro was significantly inhibited in Cezanne knockdown cells compared with cells transfected with negative control shRNA. To further verify the role of Cezanne in regulating cell growth, we next examined the effect of Cezanne on cell proliferation in vivo (**Figure 4D-G**). A549 cells infected with lentivirus containing sh-Cezanne or sh-vector as a control were subcutaneously injected into nude mice. As shown in **Figure 4D-G**, decreased Cezanne expression in A549 cells slowed down the tumor growth and reduced tumor weight in BALB/c-nu mice. Therefore, Cezanne downregulation significantly inhibited growth of A549 xenografts. These results demonstrated that Cezanne overexpression promoted NSCLC tumor progression through the hyperactivation of cell proliferation signaling.

Cezanne regulated IGF-1R signaling pathways

We further investigated the effects of Cezanne on IGF-1R signaling pathways at the stimulation of IGF-1. As a full agonist, IGF-1 could induce IGF-1R phosphorylation and downstream signaling pathways activation within 5 min. The activation effects of IGF-1 was showed as a time-dependent pattern, peaking around 10 to 30 min and lasting for at least 60 min. Intriguingly, we found phosphorylation level of IGF-1R and AKT (Ser473) increased when Cezanne was overexpressed and decreased when Cezanne was knocked down, while phospho-ERK (T202/Y204) displayed contrary results (**Figure 5A**, **5B**). Consistent with the trend of phospho-AKT (Ser473) and phospho-ERK (T202/Y204) upon Cezanne overexpression in A549 cells, similar results could also be observed in H1299 cells (**Figure 5C**). In addition, Cezanne regulated the activation of JNK and p38 following IGF-1 stimulation. Knockdown of Cezanne suppressed phospho-JNK (Thr183/Tyr185) and activated phospho-p38 (T180/Y182) in A549 cells (**Figure 5D**). Taken together, these findings showed that Cezanne activated AKT and JNK signaling pathway through enhancing IGF-1R stabilization during cell growth signaling transduction induced by IGF-1 (**Figure 7**).

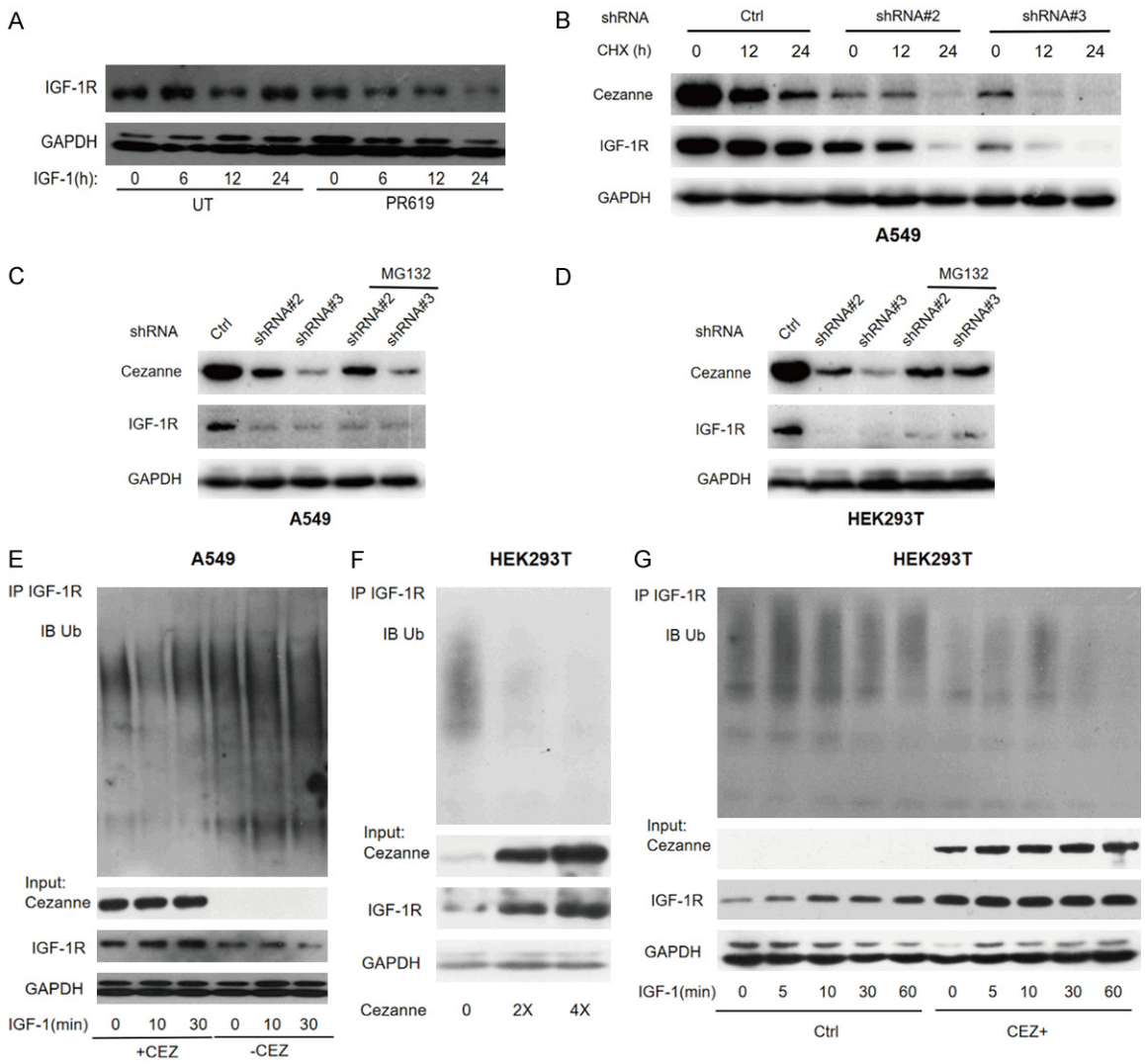

Figure 3. Cezanne stabilized and deubiquitinated IGF-1R. A. A549 cells were starved for 12 hours and treated with PR-619 for 12 hours before stimulate with 50 ng/ml IGF-1 for 0, 6, 12, 24 hours respectively. IGF-1R was analyzed by western blot. UT, untreated by PR-619. B. A549 cells transfected with lentivirus carrying sh-Cezanne or sh-Control were treated with cycloheximide (CHX) (50 μM/ml) for 0, 12, 24 hours. Then Cezanne and IGF-1R were detected by western blot. C. A549 cells transfected with lentivirus containing sh-Cezanne or sh-Control as a control were treated with MG132 (0.1 mg/ml) for 6 hours. Then Cezanne and IGF-1R were detected by western blot. D. HEK293T cells transfected with lentivirus carrying sh-Cezanne or sh-Control were treated with MG132 (0.1 mg/ml) for 6 hours. Then Cezanne and IGF-1R were analyzed by western blot. E. A549 cells were transfected with Cezanne plasmid or shRNA, starved for 12 h and treated with 50 ng/ml IGF-1 for 0 min, 10 min, 30 min, repectively. Cell lysates were subjected to immunoprecipitation (IP) and immunoblotting (IB) or directly electrophoresed with indicated antibodies. F. HEK293T cells were transfected with Cezanne plasmid. The effect of Cezanne overexpression on IGF-1R deubiquitination was analyzed by western blot. G. HEK293T cells were transfected with Cezanne plasmid, starved for 12 h, treated with 50 ng/ml IGF-1 for 0 min, 5 min, 10 min, 15 min, 30 min. Cell lysates were subjected to immunoprecipitation (IP) and immunoblotting (IB) or directly electrophoresed with indicated antibodies.

Cezanne promoted cell proliferation via IGF-1R signaling pathways

To examine the role of IGF-1R activation on hyperactivation of cell proliferation signaling mediated by Cezanne overexpression, we then performed cell proliferation assay and colony formation assay. A549 cells were transfected with Cezanne shRNA plasmid and corresponding control plasmid, with or without stimulation of IGF-1. Compared to the control, cell growth of A549 cells in Cezanne knockdown group was significantly inhibited, but was improved obviously when simulated with IGF-1 (**Figure**

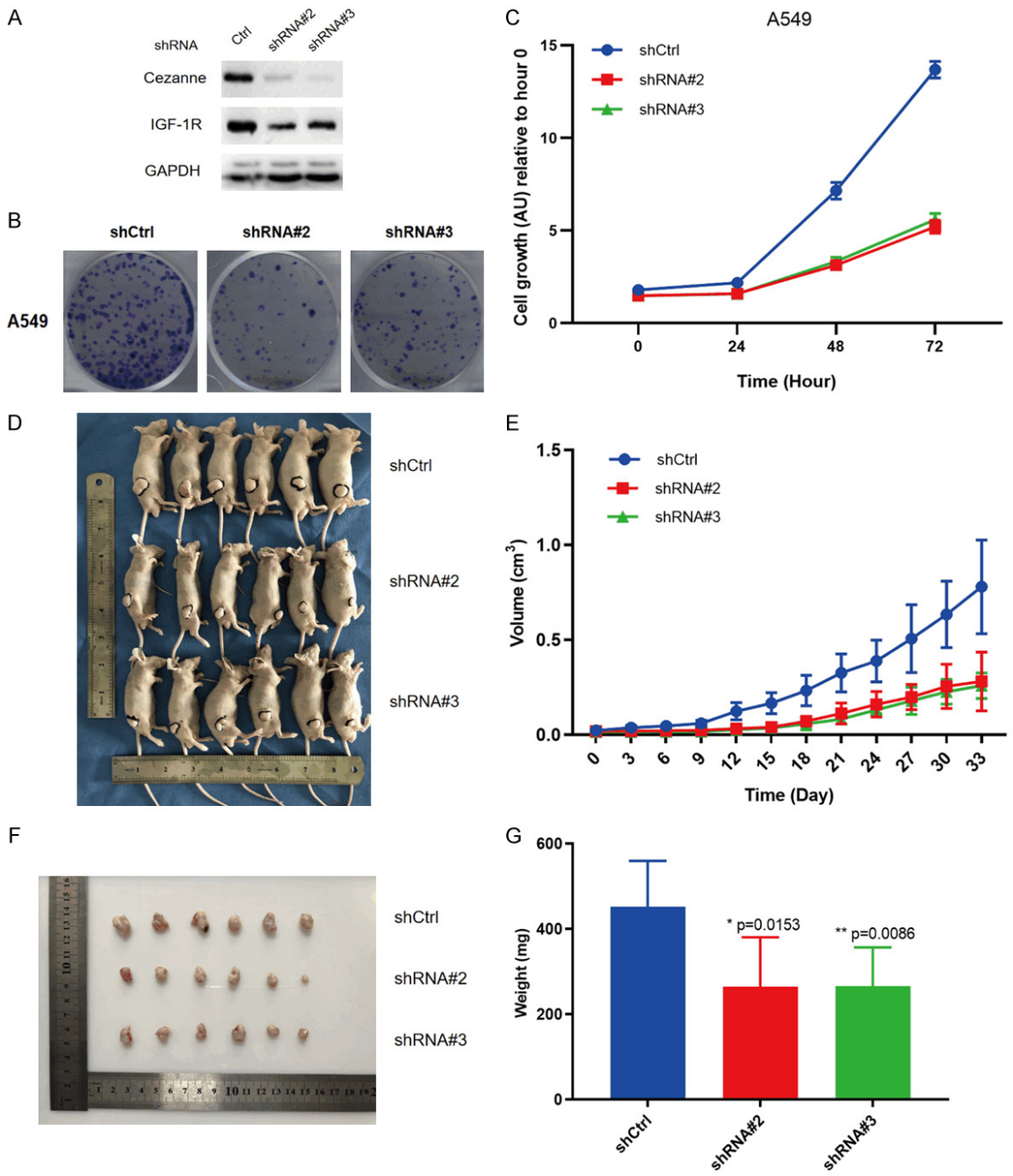

Figure 4. Knockdown of Cezanne suppressed tumor cell proliferation in vitro and in vivo. A. A549 cells used to generate tumor-bearing xenografts: Cezanne and IGF-1R were detected by western blot. B. A549 cells infected with lentivirus containing sh-Cezanne or sh-Control were used for colon formation assay. C. A549 cells infected with lentivirus were used for cell proliferation assay. D. A549 cells, infected with lentivirus containing sh-Cezanne or sh-Control, at a concentration of 2×10^6 mixed with 50% Matrigel (BD Biosciences) were implanted subcutaneously in 6-week-old male nude mice. Mice were sacrificed at 40 days after injection with no tumor volume reaching 2000 mm^3. E. Tumor volume after the day volume reached approximately 100 mm^3. F. Tumor samples separated surgically from mice. G. Tumor weight of samples separated surgically from mice, Ctrl vs. shRNA#2, *P<0.05, Ctrl vs. shRNA#3, **P<0.01. The statistical analyses were performed via the ANOVA.

6A, **6C**). We verified this result that activation of IGF-1R signaling pathway could improve the decreased cell proliferation caused by Cezanne knockdown. Further, overexpression plas-

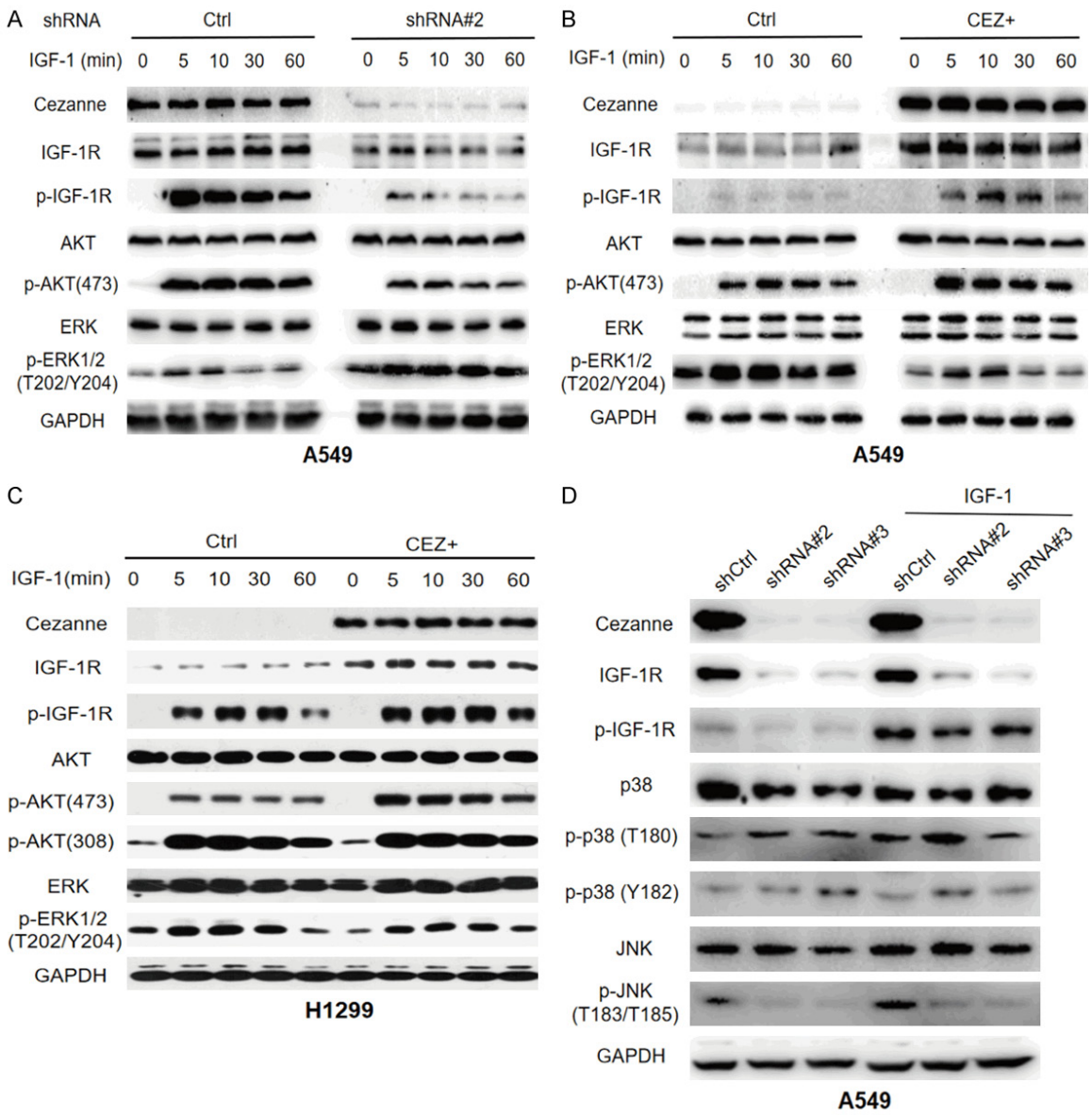

Figure 5. Cezanne regulated IGF-1R signaling pathways. A, C. A549 and H1299 cells were transfected with lentivirus containing sh-Cezanne or sh-Control, starved for 12 h, then treated with 50 ng/ml IGF-1 for 0, 5, 10, 30, 60 min. B. A549 cells were transfected with Cezanne plasmid or empty vector as a control, starved for 12h, then treated with 50 ng/ml IGF-1 for 0, 5, 10, 30, 60 min. A-C. Cezanne, IGF-1R, AKT, ERK, p-IGF-1R, p-AKT and p-ERK were analyzed by western blot. D. A549 cells were transfected with lentivirus carrying sh-Cezanne or sh-Control, starved for 12 h, then treated with 50 ng/ml IGF-1 for 10 min. Cezanne, IGF-1R, p-IGF-1R, p38, p-p38, JNK and p-JNK were detected by western blot.

mid of Cezanne and empty vector plasmid were transfected into H1299 cell line. Similar results could also be observed in H1299 cells transfected with Cezanne overexpression plasmid (**Figure 6B**, **6D**). Overexpression of Cezanne could promote cell proliferation in H1299 cells, and stimulation of IGF-1 had the similar but not equal effect on cell proliferation. These results revealed that Cezanne promoted tumor progression through IGF-1R signaling activation (**Figure 7**).

Discussion

Lung cancer is the most common malignancy with high mortality worldwide [33]. Despite recent advances in understanding its tumorigenesis and progression, and introduction of new

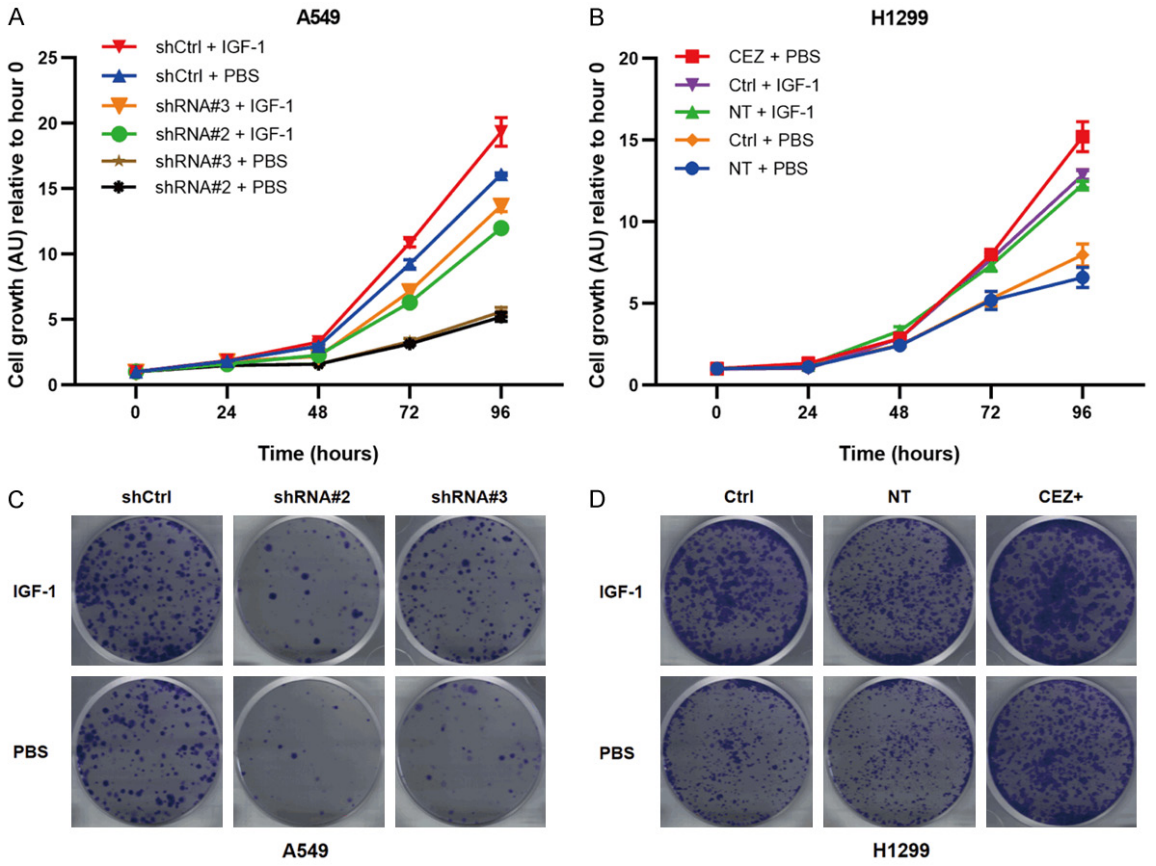

Figure 6. Cezanne promoted cell proliferation via IGF-1R signaling pathways. A. A549 cells were transfected with lentivirus carrying sh-Cezanne or sh-Control, seeded in 96-well plates at a density of 1000 cells per well, treated with IGF-1 or PBS, and then performed with cell proliferation assay. B. H1299 cells were transfected with Cezanne overexpressing plasmid or empty vector as a control, seeded in 96-well plates at a density of 1000 cells per well, treated with IGF-1 or PBS, and then performed with cell proliferation assay. C. A549 cells, transfected with lentivirus carrying sh-Cezanne or sh-Control, were seeded in 6-well plates at a density of 1000 cells per well, treated with IGF-1 or PBS, and then performed with colony formation assay. D. H1299 cells, transfected with Cezanne overexpressing plasmid or empty vector as a control, were seeded in 6-well plates at a density of 1000 cells per well, treated with IGF-1 or PBS, and then performed with colony formation assay.

diagnostic and therapeutic strategies to lung cancer, the 5-year survival rate remains less than 15% [34, 35]. Insulin-like growth factor-I receptor (IGF-IR), a receptor tyrosine kinase, is overexpressed and closely related to tumor cell transformation, growth, proliferation and prevention of apoptosis in various cancers including NSCLC. However, clinical studies suggest that therapies targeting IGF-1R can hardly improve the outcomes of patients with obvious side effects [36]. As a consequence, considerable effort has been devoted to identifying oncogenic signaling pathways that can be therapeutically targeted. Recently, DUBs has become a new therapeutic option against cancer [37]. Our previous immunohistochemistry (IHC) analyses found that IGF-1R was positively related to Cezanne [32], a member of ovarian tumor domain deubiquitinase. Herein, we identify Cezanne as a crucial regulator of cell proliferation that controls IGF-IR signaling pathways via deubiquitination of IGF-1R.

Cezanne likely deubiquitinates and stabilizes IGF-IR through its active deubiquitinating domain and ubiquitin-associated domain (UBA), leading to the activation of downstream AKT and JNK pathway. This notion is supported by a systematic examination. Compared to control group, higher expression level of IGF-1R is detected by western blot in cells transfected with Cezanne overexpression plasmid, while IGF-1R is down-regulated in Cezanne knockdown group. In co-IP assay, Cezanne is show-

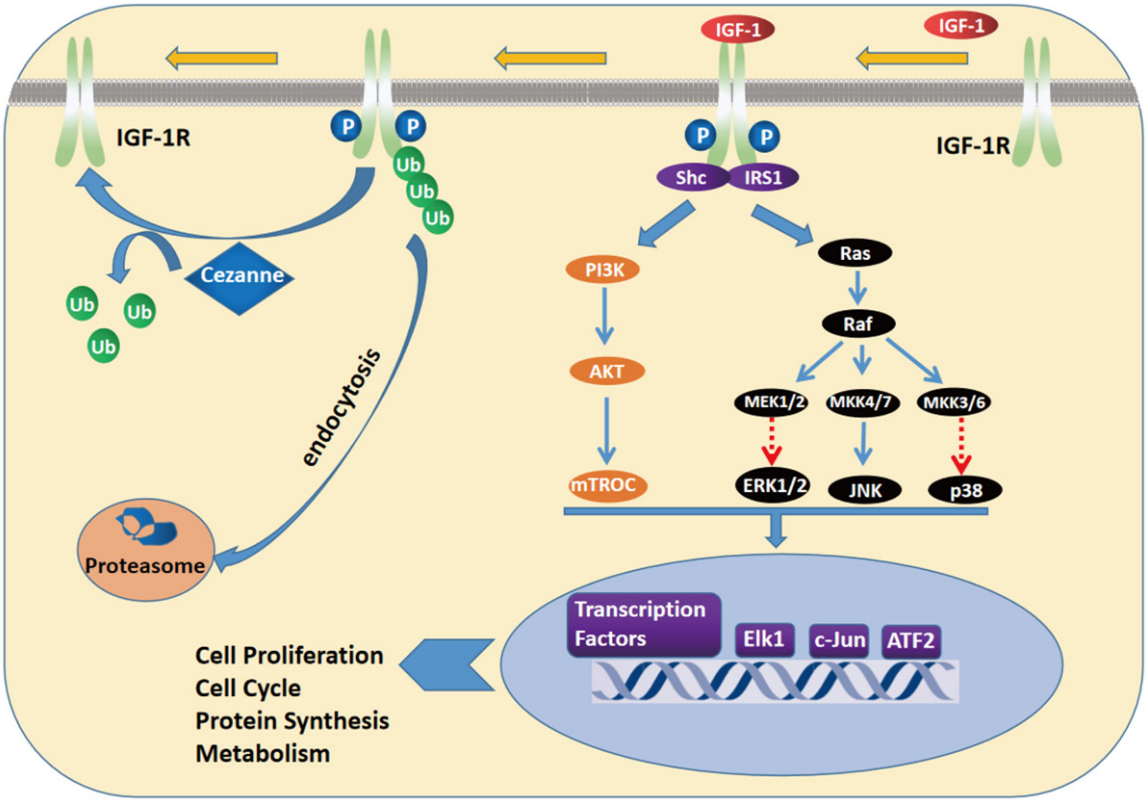

Figure 7. Cezanne deubiquitinated IGF-1R and regulated IGF-1R signaling pathways. Cezanne directly targeted IGF-1R by deubiquitination and stabilization. This leaded to activation of downstream AKT and JNK, which bolstered tumor cell growth. These findings revealed Cezanne as a regulator of tumor cell proliferation via IGF-1R signaling pathway and a potential target for NSCLC therapy.

ed to physically interact with IGF-1R and negatively regulate the ubiquitination level of IGF-1R in vitro. The oncogenic role of Cezanne in NSCLC is further proved both in vivo and in vitro. Moreover, Cezanne stabilizes IGF-1R and suppresses the ubiquitination level of IGF-1R.

Several studies demonstrate that Mdm2, Nedd4 and c-Cbl are important E3 ligases to mediate the ubiquitination of IGF-1R and that IGF-1R is most likely ubiquitinated before entering into endocytic vesicle [15-17]. However, seldom studies investigate the deubiquitination of IGF-1R. Cezanne, which possesses an active deubiquitinating domain (OTU), a ubiquitin-associated domain (UBA) and an A20-like zinc finger, serves as DUB in signal transduction. And it has been discovered to regulate NF-κB signaling [25, 28], EGFR trafficking [26], and homeostasis of HIF-1α/HIF-2α [30, 31]. Mevissen T.E. reveals that Cezanne tends to specifically target Lys11-linked polyubiquitin [27]. While Bin Wang claims that Cezanne, apart from hydrolyzing K11 ubiquitin chains, is also a bona fide DUB that removed K63-linkage polyubiquitination from GβL. Though there is currently no report that IGF-1R can be ubiquitinated via K11 ubiquitin chains. Cezanne is supposed to deubiquitinate IGF-1R through hydrolyzing some other kinds of ubiquitin chains, and further study is needed to discover the exact mechanism.

Further experiments reveal that Cezanne promotes tumor progression through the hyperactivation of cell proliferation signaling mediated by IGF-1R signaling pathways. Phosphorylation level of IGF-1R and AKT (Ser473) are found to upregulate in Cezanne overexpression group, but there is no obvious change in phospho-AKT (Thr308). Phosphorylation of AKT at Thr308 of the activation loop is mediated by PDK1 [38], while phosphorylation of Ser473 in the hydrophobic loop is mainly caused by mTORC2 [39]. AKT also activates mTORC1, which is an essential hub integrating extracellular stimuli

and nutrient signals to regulate cell growth [40]. Cezanne-IGF-1R axis is supposed to regulate AKT activation mainly through AKT-mTOR loop. Precise control of PI3K/AKT/mTOR signaling is crucial for normal cell proliferation, and its aberrant activation leads to a number of pathological outcomes, including cancers.

Phospho-JNK (Thr183/Tyr185) is shown to downregulate upon Cezanne knockdown. JNK signaling pathway mediates a wide range of cellular processes, including cell proliferation, survival, and migration, as well as cell apoptosis, senescence, and stress responses [41]. In contrast to JNK, phospho-p38 (T180/Y182) and phospho-ERK1/2 (T202/Y204) increase in Cezanne knockdown cells upon IGF-1R activation. Originally, p38 is described as a tumor-suppressor kinase for its inhibitory role in RAS-dependent transformation. The occurrence of proliferation inhibition comes with suppression of IGF-1R expression, and then increases the phosphorylation of p38 MAPK. While ERK1/2 is recognized as the promotor of cell proliferation in various cancers. We suppose that the following ERK1/2 phosphorylation is caused by kind of feedback regulation of proliferation inhibition.

Conceivably, overexpression of Cezanne results in deubiquitination of surface-bound or internalized IGF-1R, thereby enhancing their functions in tumors. Considering that recycling mechanisms are shared by several growth factor receptors, such as EDFR and IGF-1R, Cezanne is also expected to enhance recycling of multiple endosomal cargos, thereby promoting tumor progression. Nonetheless, the locus of Cezanne targeting IGF-1R for deubiquitination is still not clear and urgent to be explored. More efforts are supposed to be focus on investigating the potential pharmacological strategies to intercept Cezanne in tumors.

In summary, the present investigation unveils that Cezanne targets IGF-1R for deubiquitination and stabilization, thereby AKT/JNK activation, suggesting crosstalk between Cezanne and IGF-1R signaling function as a common promoting mechanism to enhance cell proliferation in NSCLC progression. Therefore, it is of great interest to develop therapeutic approaches targeting Cezanne concurrently with IGF-1R inhibition for potential cancer interventions.

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Disclosure of conflict of interest

None.

Abbreviations

IGF-1R, insulin-like growth factor 1 receptor; NSCLC, non-small-cell lung cancer; OTUD, ovarian tumor domain; AKT, serine/threonine protein kinase; PI3K, phosphoinositide 3-kinase; JNK, C-Jun N-terminal kinase; ERK, extracellular signal-regulated kinase; IHC, Immunohistochemistry; PM, plasma membrane; qRT-PCR, quantitative real-time polymerase chain reaction; co-IP, co-Immunoprecipitation.

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