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RESEARCH PAPER

RING finger gene 180 inhibits osteosarcoma progression through regulating chromobox homolog 4 ubiquitination

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ABSTRACT

Osteosarcoma (OS) is still the most common malignant bone tumor whose etiology remains largely unclear. Here, we aimed to investigate the role of a novel E3 ubiquitin ligase RING finger gene 180 (RNF180) in OS progression. RNF180 was significantly down-regulated in both OS tissues and cell lines. We up-regulated RNF180 using over-expression vector and knocked down RNF180 using specific short hairpin RNAs in OS cell lines. RNF180 over-expression inhibited the viability and proliferation yet promoted apoptosis in OS cells, while RNF180 knockdown showed the opposite effects. RNF180 also suppressed tumor growth and lung metastasis in mouse model, accompanied with elevated E-cadherin level and decreased ki-67 level. Besides, chromobox homolog 4 (CBX4) was predicted as a substrate of RNF180. RNF180 and CBX4 were both localized mainly in nucleus and their interaction was validated. RNF180 aggravated the decline of CBX4 level after cycloheximide treatment. RNF180 also promoted the ubiquitination of CBX4 in OS cells. Furthermore, CBX4 was significantly up-regulated in OS tissues. RNF180 also up-regulated Kruppel like factor 6 (KLF6) yet down-regulated RUNX family transcription factor 2 (Runx2) in OS, which served as downstream targets of CBX4. In addition, RNF180 inhibited migration, invasion and epithelial-mesenchymal transition (EMT) in OS cells, which were partially abolished by CBX4 over-expression. In conclusion, our findings demonstrated that RNF180 inhibits OS development via regulating CBX4 ubiquitination, and RNF180-CBX4 axis is a potential therapeutic target for OS treatment.

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Osteosarcoma; RNF180; CBX4; ubiquitination; migration and invasion

Introduction

Osteosarcoma (OS) remains the most frequent primary malignant bone tumor with an overall incidence of 2–3 million/year. OS mainly originates from long bones including distal femur, proximal tibia, and humerus [1]. Children, adolescents, and young adults are most vulnerable to OS with a probable cause of pubertal growth spurt. Radiation and alkylating agents exposure are also risk factors for OS development [1,2]. Persistent pain and localized swelling are major symptoms of OS which seriously affects the normal life of OS patients. Although the overall five-year survival for localized OS has reached 78% owing to the combination of surgical resection, radiation, and multi-agent chemotherapy, survival rate for patients with metastasis or relapse remains only 20% [2–4]. Therefore, a deeper understanding of the underlying mechanisms and exploring new therapeutic targets are of great significance for improving current OS treatment strategy.

RING finger gene 180 (RNF180), also known as Rines, is a novel E3 ubiquitin ligase containing a RING finger domain for ubiquitin ligase activity and a basic coiled-coil domain [5]. E3 ubiquitin ligases play a vital role in the determination of ubiqutin-mediated protein degradation, and are further involved in physiological processes including DNA repair, cell proliferation and differentiation [6,7]. Increasing evidence demonstrated that RNF180 is aberrantly expressed in a variety of human cancers including gastric cancer, lung cancer, and colorectal cancer, and high-level methylation of RNF180 promoter predicts metastasis and poor survival [8–11]. However, the role that RNF180 plays in tumorigenesis and development of OS remains undisclosed. Expression level of RNF180 is significantly down-regulated and its

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promoter methylation level is elevated in sarcoma tissues according to UALCAN online database (http://ualcan.path.uab.edu/index.html), indicating that RNF180 might be implicated in OS development [12]. Chromobox homolog 4 (CBX4) is a member of CBX protein family which acts as a both tumor repressor and oncogene in human cancers [13,14]. CBX4 is significantly up-regulated in sarcoma tissues and is predicted to be a downstream target of RNF180 on Ubibrowser database (http://ubibrowser.ncpsb.org.cn). Besides, CBX4 exerts oncogenic effect on OS progression and promotes metastasis via targeting downstream Kruppel like factor 6 (KLF6) in clear cell renal cell carcinoma and RUNX family transcription factor 2 (Runx2) in OS [15–17]. KLF6 was studied to be downregulated in OS and could hinder OS cell proliferation and invasion [18,19], while Runx2 is highly expressed in OS cells and related to bone development [20,21].

Here in this study, we detected the expression of RNF180 in OS, and investigated the effect of RNF180 on proliferation, migration, and invasion of OS. We also validated the interaction between RNF180 and CBX4, and explored their combined effects on OS development.

Materials and methods

Clinical tissues and cells

72 pairs of OS and adjacent normal tissues were obtained after surgery with consent of all patients. Sample collection and subsequent quantitative real-time PCR and immunohistochemistry assay were carried out following the Declaration of Helsinki and approved by the First Clinical Hospital affiliated to Harbin Medical University. HEK293T cells, human osteoblast cell line hFOB 1.19, and four OS cell lines (MnnG/HOS, Saos-2, MG-63, and U2OS) were purchased from Procell (China). Cells were transfected with plasmids or short haipin RNAs (shRNAs) using lipofectamine2000 (Thermofisher Scientific, USA) according to the instruction of manufactures. Stable transfected cells were selected by resistance to neomycin. RNF180 was inserted into pcDNA3.1 plasmid between BamH I and Xho I for over-expression; for subsequent animal experiment, EGFP-tag was added for in vivo imaging. CBX4 was inserted into pcDNA3.1 between BamH I and Xba I. shRNAs for RNF180 (sh1: 5'-GCATTAATCAGAGGCTTAA-3'; sh2: 5'- GGATGGATTACCTGCACTT-3') were directly synthesized by Genepharma (China).

Quantitative real-time PCR

Total RNA in OS/normal adjacent tissues ($n = 72$) and OS/osteoblast cell lines was extracted using TRIZOL lysis buffer (Thermofisher Scientific, USA), and reversely transcribed into cDNA using SuperScript™ II Reverse Transcriptase (Thermofisher Scientific, USA). Relative RNA levels of RNF180 and CBX4 were then quantified using Maxima SYBR Green/ROX (Thermofisher Scientific, USA) and data was analyzed utilizing $2^{-\Delta\Delta}$ method. Primers for real-time PCR are listed in Table 1.

Methylation-specific PCR

For Methylation-specific PCR (MSP), total genomic DNA in tissues and cell lines was extracted using GeneJET Genomic DNA Purification Kit (Thermofisher Scientific, USA). Then genomic DNA was treated with bisulfite to analyze methylation level using DNA Methylation-Gold kit (ZYMO RESEARCH, USA), followed by subsequent PCR detection. Primers used in MSP are listed in Table 2.

Western blotting

Total protein in OS tissues ($n = 5$) and cell lines was extracted using RIPA lysis buffer (Thermofisher Scientific, USA), followed by quantification using BCA protein assay kit (Abcam, UK). Then total protein was fractionated by sodium dodecyl sulfate polyacrylamide gel electrophoresis and transferred onto PVDF membranes (ThermoFisher Scientific). The membranes were

Table 1. Primers for real-time PCR.

Target	Primers
RNF180	Forward: 5′-TCTGACTTTCCTGATGGACCTG-3′
	Reverse: 5′-CCTGAGTATTTACCCTGCTTCTGT-3′
CBX4	Forward:5′-GCAGAGTGGAGTATCTGGTGA-3′
	Reverse: 5′-AGCTTGGCACGGTTGTCAG-3′
GAPDH	Forward: 5′-CAGCCTCAAGATCATCAGCA-3′
	Reverse: 5′-TGTGGTCATGAGTCCTTCCA-3′

Table 2. Primers used in MSP.

Target	Primers
RNF180-methylated	Forward: 5′-TTTGCGCGGGGTTAAAGTTC-3′
	Reverse: 5′-CGATACCGATTCGACGAAACG-3′
RNF180-Unmethylated	Forward: 5′-TGTTTGTTTGTGTGGGGTTAAAGTTT-3′
	Reverse: 5′-CAACAACAATACCAATTCAACAAAACA-3′

then sealed with 5% milk powder and incubated with diluted primary antibodies overnight at 4°C as follow: rabbit anti-RNF180 (1:1000, Thermofisher Scientific, USA), mouse anti-CBX4 (1:1000, Thermofisher Scientific, USA), mouse anti-KLF6 (1:1000, Thermofisher Scientific, USA), rabbit anti-Runx2 (1:2000, Thermofisher Scientific, USA), and mouse anti-GAPDH (1:2000, Thermofisher Scientific, USA). The membranes were rinsed using TBS buffer for three times and incubated with HRP-conjugated goat anti-mouse/rabbit secondary antibody (1:10000, Thermofisher Scientific, USA) for 1 hour at room temperature. Target proteins were visualized using Enhanced chemiluminescence (ECL) reagents (Beyotime, China), and analyzed using Gel-Pro-Analyzer (Media Cybernetics, USA).

Immunohistochemistry

Paraffin-embedded OS tissues were subjected to de-waxing and rehydrating, and boiled with 10 mM sodium citrate buffer for 10 minutes. Then tissue sections were treated with 3% H_2O_2, and incubated with primary antibodies at 4°C overnight, followed by incubation with HRP conjugated Goat anti-Rabbit secondary antibody. Afterwards, tissue sections were stained with DAB substrate (Solarbio, China) for five minutes and observed under a microscope (200×, Olympus, Japan). Primary antibodies used were as follow: Rabbit anti-RNF180, Rabbit anti-CBX4, Rabbit anti-E-cadherin, Rabbit anti-N-cadherin, Rabbit anti-Ki67 (1:200, Thermofisher Scientific, USA).

Cell counting kit-8 (CCK-8) assay

U2OS and Saos-2 cells were cultured on a 96-well plate after transfection, and cultured with 10 μL CCK-8 solution (Beyotime, China) for an hour. Then optical density at 450 nm was recorded at 0 h, 24 h, 48 h, 72 h, and 96 h after treatment.

Clone formation assay

U2OS and Saos-2 cells at logarithmic phase were seeded onto 6-well plates. After transfection with plasmids or shRNAs, cells were cultured for 15 d until colonies were visible. Then cells were fixed with 4% paraformaldehyde for 15 minutes and stained with GIMSA for 30 minutes, followed by cell colonies counting (>50 cells).

5-Ethynyl-2′-deoxyuridine (Edu) staining

Edu-staining was performed using EdU Cell Proliferation Kit (Beyotime, China). In brief, U2OS and Saos-2 cells were incubated with 10 μM Edu for 2 hours, fixed with 4% paraformaldehyde for 15 minutes and permeated with 0.3% Triton X-100 for 5 minutes. After staining with DAPI, cell slides were sealed with anti-quenching reagent and typical images were captured under a microscope (200×).

6-TdT-mediated dUTP Nick-End Labeling (TUNEL) staining

U2OS and Saos-2 cells were fixed using 4% paraformaldehyde for 30 minutes, washed with PBS buffer and permeated using 0.3% Triton X-100 for 5 minutes. Then cells were stained using one-step TUNEL Apoptosis Assay Kit (Beyotime, China) according to instructions, followed by staining with DAPI for 5 minutes. Afterwards, cell slides were blocked using anti-quenching reagent and observed under a microscope at 200 ×magnification.

Wound healing and Transwell assay

For wound healing assay, U2OS and Saos-2 cells were seeded onto 6-well plates and cultured to confluence. A straight cell scratch was made using a 200 μL pipette tip and typical images were captured at 0 h and 24 h scratch. For transwell assay, cells were seeded on the upper layer inserted with Matrigel, and cultured with serum-

free medium for 48 h. Medium containing 20% fetal bovine serum was added onto lower chamber. Cells migrated to lower chamber were stained with 0.1% crystal violet for 10 minutes and counted under a microscope.

Immunofluorescence assay

The intracellular expression of E-cadherin and N-cadherin expression, co-localization of RNF180 and CBX4 were detected using Immunofluorescence assay. Similar to TUNEL assay, cells were fixed with 4% paraformaldehyde and permeated using 0.3% Triton X-100 for 5 minutes, followed by blockade using goat serum for 30 minutes. Then cells were incubated with primary antibodies overnight at 4°C: mouse anti-E-cadherin, mouse anti-N-cadherin, rabbit anti-Myc or mouse anti-Flag (1:500, Abcam, UK). Afterwards, cells were rinsed and incubated with secondary antibodies for an hour away from light: Goat anti-Rabbit lgG Alexa Flour 488, Goat anti-mouse lgG Alexa Flour 488, Goat anti-mouse lgG Alexa Flour 594 (1:500, Abcam, UK). Then cells were sealed with anti-quenching reagent and observed under a microscope.

Co-immunoprecipitation assay (Co-IP)

HEK293T cells were co-transfected with flag tagged RNF180 and myc tagged CBX4 for 36 hours. Afterwards, cells were lysed using Pierce^{tm} IP buffer (Thermofisher Scientific, USA) supplemented with protease inhibitor, followed by incubation with rabbit anti-Myc (1:100, Abcam, UK) or mouse anti-Flag (1:100, Abcam, UK) for four hours at 4°C. Then the tag-antibody complex was incubated with Protein A agarose beads (Cell signaling technology, USA) for another four hours, followed by subsequent western blotting detection using anti-Fag and anti-Myc primary antibody. For ubiquitin level detection, U2OS and Saos-2 cells were transfected with Flag-RNF180, Myc-CBX4, and Rabbit anti-HA (1:100, Abcam, UK), followed by western blotting detection using anti-Myc. For ubiquitination of CBX4 detection, cells were treated with 10 μM MG132 for an hour before Co-IP.

Xenograft tumor assay and in vivo imaging

Healthy 6-week-old balb/c node mice were purchased from Guangdong medical animal center (China) and housed in a 12 h day/night cycle chamber for a week. Mice were randomly divided into four groups and injected with OS cells via tail vein (3×10 [5] cells every mouse): RNF180 over-expressed U2OS, RNF180 over-expressed Saos-2 cells, U20S cells transfected with vector and Saos-2 cells transfected with vector. Tumor metastasis was observed using an in vivo imaging system (PerkinElmer, USA) every week for three weeks. Then mice were euthanized by intraperitoneal injection of barbital sodium (200 mg/kg), tumor weight was recorded and tumors were subjected to subsequent hematoxylin-eosin staining. All animal experiments were performed following the guide for the care and use of laboratory animals and approved by the First Clinical Hospital affiliated to Harbin Medical University.

Hematoxylin-eosin (H&E) staining

Lung tissues of mice were paraffin embedded and sliced into 5 μm sections, followed by deparaffinating and dehydrating. Then tissue sections were stained with hematoxylin (Solarbio, China) for 15 minutes, and stained with eosin (Solarbio, China) for 2 minutes after washing. Tissue sections were then washed and subjected to dehydrating and sealing with neutral balsam, lung metastasis nodules were counted under a microscope at 200× magnification.

Statistical analysis

All experiments were repeated for more than three times and data were presented as means ± standard deviation. Data analysis was performed using Graphpad Prism 8.0, differences were compared using unpaired t-test (between two groups) or one-way ANOVA (three or more groups). Two-way ANOVA was used to analyze results from CBX expression after cycloheximide treatment, CCK-8 assay, and tumor volume. $P < 0.05$ was considered to have a statistical significance.

Results

RNF180 displays lower expression and promoter hypermethylation in OS

First of all, we analyzed RNF180 expression and promoter methylation in sarcoma on UALCAN database (http://ualcan.path.uab.edu/) based on race, gender, age, and TP53 mutation status. Generally, RNF180 expression level was significantly down-regulated in sarcoma tissues while promoter methylation level was obviously elevated (Figure 1a). Then 72 pairs of OS and adjacent normal tissues were collected, the RNA level of RNF180 was significantly down-regulated in OS tissues compared to normal control (Figure 1b). Further western blotting and IHC assays showed that the protein level of RNF180 is obviously lower in OS tissues in contrast with normal tissues (Figure 1c,d). Besides, MSP was used to detect promoter methylation on RNF180, unmethylated bands were observed in all normal tissues, and two tissues displayed methylated bands. On the contrary, methylated bands were detected in all OS tissues and unmethylated bands were nearly undetectable (Figure 1e,f). Similarly, methylation was observed in OS cell lines yet undetectable in normal human osteoblast cell (Figure 1g). These results indicated that RNF180 displayed lower expression and higher promoter methylation level in OS tissues.

RNF180 inhibits OS cell proliferation in vitro

Four OS cell lines showed significantly lower RNF180 protein level compared to hFOB1.19 cells, among which U2OS and Saos-2 cells displayed medium RNF180 level and were chosen for subsequent over-expression and knockdown assays (Figure 2a). RNF180 level was significantly elevated after pcDNA3.1-RNF180 transfection and

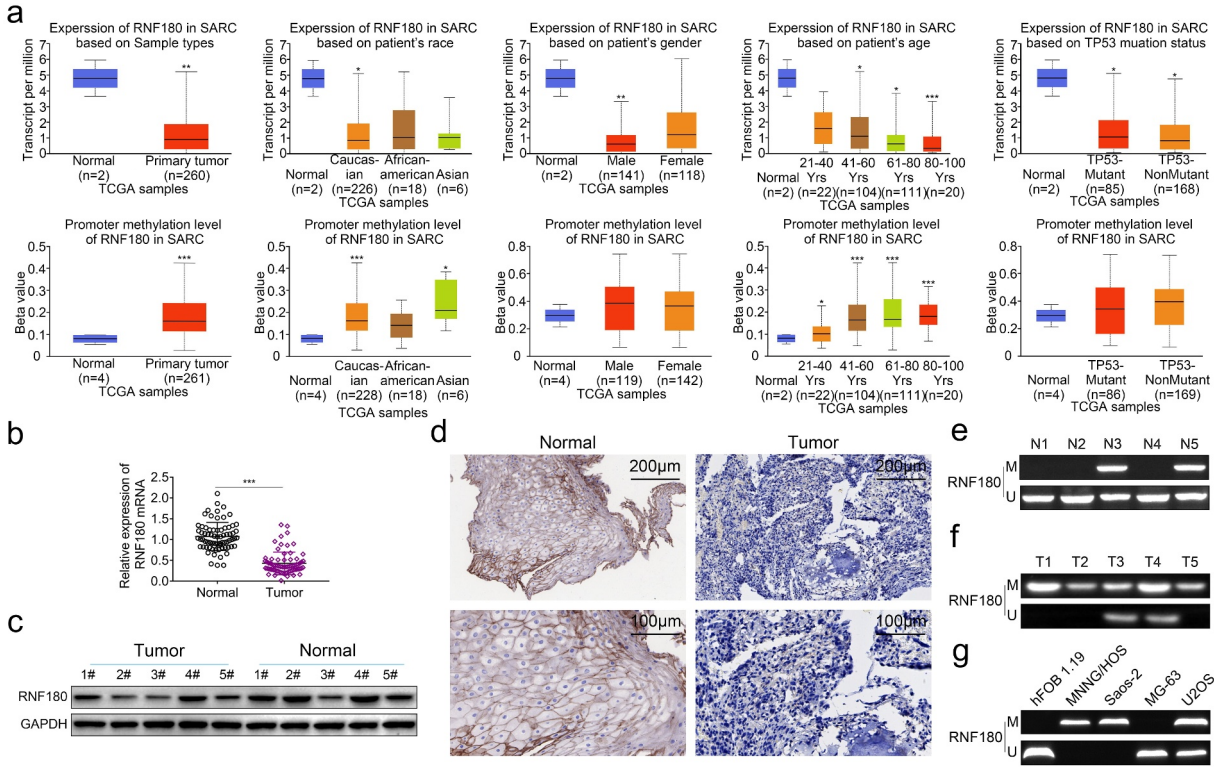

Figure 1. rnf180 displays lower expression and promoter hypermethylation in OS. (a) The expression and promoter methylation level of RNF180 in sarcoma tissues based on race, gender and TP53 mutation status were available on UALCAN database. (b) the RNF180 mRNA level in OS and adjacent normal tissues was measured using quantitative real-time PCR ($n = 72$). (c, d) the protein level of RNF180 in OS and adjacent normal tissues was detected by western blotting and immunohistochemistry (bar = 200 μm, 100 μm) assays. (e, f) Methylation-specific PCR was used to evaluate methylation level in OS tissues ($n = 5$) and cell lines. (g) Methylation-specific PCR was used to evaluate methylation level in human osteoblast cells (hFOB1.19) and OS cells (MnnG/HOS, Saos-2, MG-63, and U2OS). OS: osteosarcoma; RNF180: Ring Finger Protein 180; *p < 0.05, **p < 0.01, ***p < 0.001.

knocked down after shRNF180 transfection in U2OS and Saos-2 cells (Figure 2b). CCK-8 assay showed that cell viability was enhanced after RNF180 knockdown yet suppressed after RNF180 over-expression in U2OS and Saos-2 cells (Figure 2c). Further clone forming assay showed that cell colony number was reduced in pcDNA3.1-RNF180 group yet significantly increased in shRNF180 group (Figure 2d). Similarly, Edu staining assay showed that Edu positive cells were decreased after RNF180 over-expression but increased after RNF180 knockdown (Figure 2e). TUNEL staining assay showed that Tunel positive cells were increased after RNF180 over-expression yet reduced after RNF180 knockdown (Figure 2e). All these results indicated that RNF180 over-expression inhibits OS cell proliferation *in vitro*.

RNF180 inhibits migration and invasion of OS cells

The relative wound width of U2OS and Saos-2 cells was significantly larger after RNF180 over-expression yet smaller after RNF180 knockdown (Figure 3a). Transwell assay showed that the numbers of U2OS and Saos-2 cells on lower chamber were remarkably decreased after RNF180 over-expression but increased after RNF180 knockdown (Figure 3b). Further immunofluorescence detection showed that the expression of E-cadherin was up-regulated in two OS cell lines after RNF180 over-expression but not detectable after RNF180 knockdown. On the contrary, the protein level of N-cadherin was decreased after RNF over-expression yet conspicuously elevated after RNF180 knockdown (Figure 3c). These results suggested that RNF180 inhibited migration and invasion in OS cells.

RNF180 is a putative E3 ubiquitin enzyme of CBX4

RNF180 was predicted as a candidate E3 ubiquitin enzyme of CBX4 on Ubibrowser database (Figure 4a). Immunofluorescence assay showed that RNF180 and CBX4 both localized in nucleus and cytoplasm in HEK293T cells, but in U2OS cells, CBX4 mainly localized in the nucleus while RNF180 distributed in the whole cells (Figure 4b). Further Co-IP showed that Myc-CBX4 is observed by western blotting after using Flag-RNF180 as a bait for IP. Similarly, Flag-RNF180 is detectable after using Myc-CBX4 as a bait for IP (Figure 4c). U2OS and Saos-2 cells were treated with cyclohexamide (CHX), and the decrease rate of CBX4 level was significantly higher after RNF180 over-expression compared with mock (Figure 4d). After IP using HA-ubiquitination tag, the protein level of Myc-CBX4 was obviously lower in MycCBX4+Flag-RNF180 co-transfected group in contrast with that in MycCBX4 group (Figure 4e). These results indicated that RNF180 interacts with CBX4 and reduces CBX expression in OS cells via ubiquitination.

RNF180 regulates KLF6 and Runx2 expression through targeting CBX4

The mRNA level of CBX4 is significantly higher in OS tissues compared with normal control (Figure 5a). Further IHC assay showed that CBX4 protein level is remarkably higher in OS tissues in contrast with normal control (Figure 5b). We further detected the CBX4 mRNA levels in U2OS and Saos-2 cells with overexpressed or silent RNF180, which however, had no significant alteration (Figure 5c). Since both KLF6 and Runx2 are two direct downstream targets of CBX4 and involved in OS progression, we also explored the KLF6 and Runx2 expression levels in OS cells with altered RNF180. It was shown that the protein levels of CBX4 and Runx2 in OS cells were significantly down-regulated after RNF180 over-expression yet up-regulated after RNF180 knockdown, while the KLF6 level showed the opposite trend (Figure 5d). Moreover, U2OS and Saos-2 cells were transfected with pcDNA3.1-RNF180 and pcDNA3.1-CBX4, the protein levels of CBX4 and Runx2 were decreased after RNF180 over-expression, while the effect was partially reversed after pcDNA3.1-CBX4 co-transfection. On the contrary, the KLF6 level was significantly elevated yet the effect was partially abolished after CBX4 over-expression (Figure 5e). These results suggested that RNF180 regulated the expression of KLF6 and Runx2 via targeting CBX4.

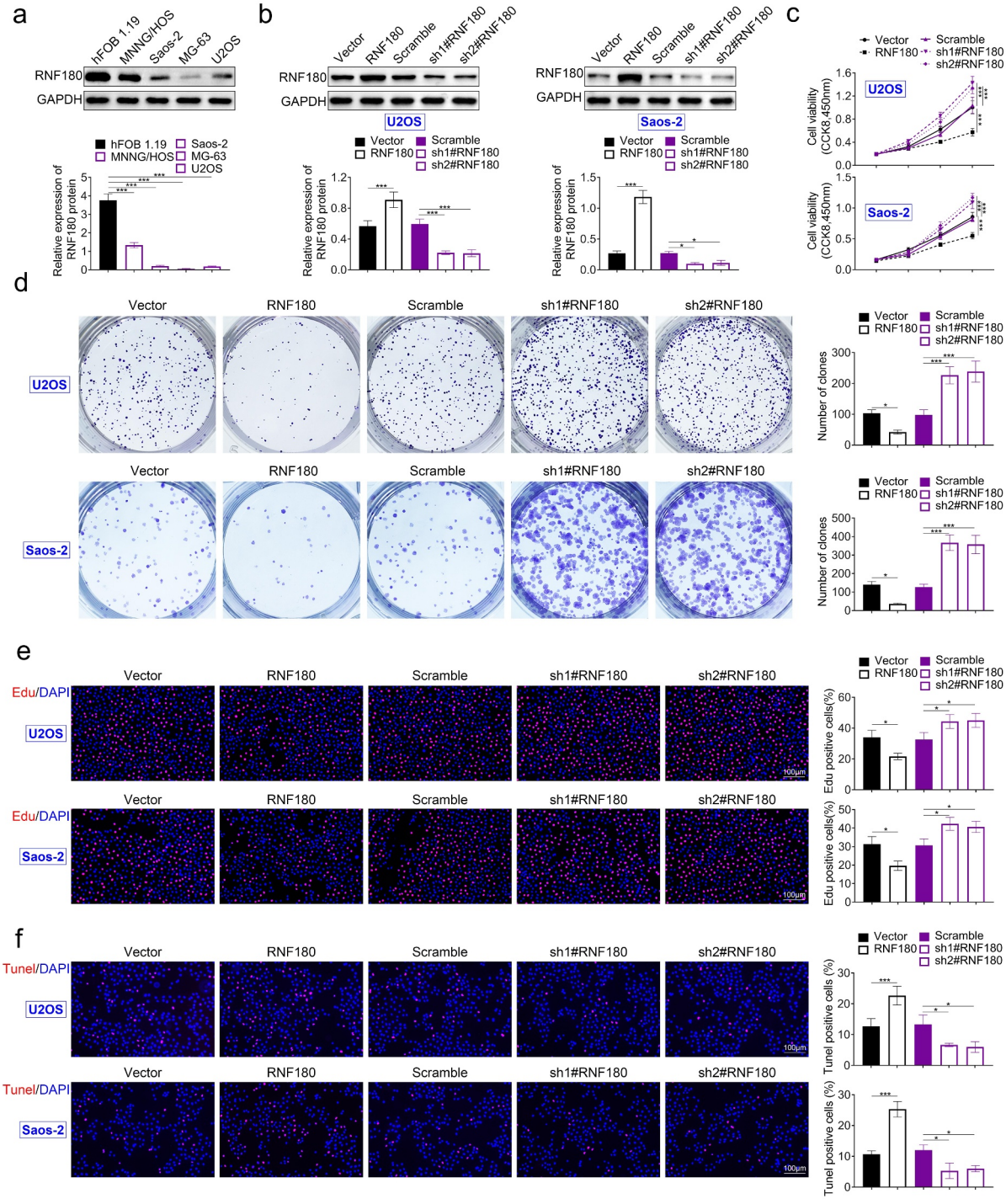

Figure 2. RNF180 inhibits OS cell proliferation in vitro. U2OS and Saos-2 cells were transfected with vector, pcDNA3.1-RNF180, scramble shRNA or RNF180 shRnas. (a) the RNF180 mRNA level in human osteoblast cell line hFOB 1.19 and four OS cell lines were measured using quantitative real-time PCR. (b) RNF180 protein level was detected using western blotting. (c) Cell viability was evaluated using a commercial cell counting kit-8. (d, e) Cell proliferation was assessed using clone forming assay and Edu staining (bar = 100 μm). (f) Cell apoptosis was detected using Tunel staining (bar = 100 μm). RNF180: Ring Finger Protein 180; Edu: Ethynyl-2′-deoxyuridine; Tunel: TdT-mediated dUTP Nick-End Labeling; *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$.

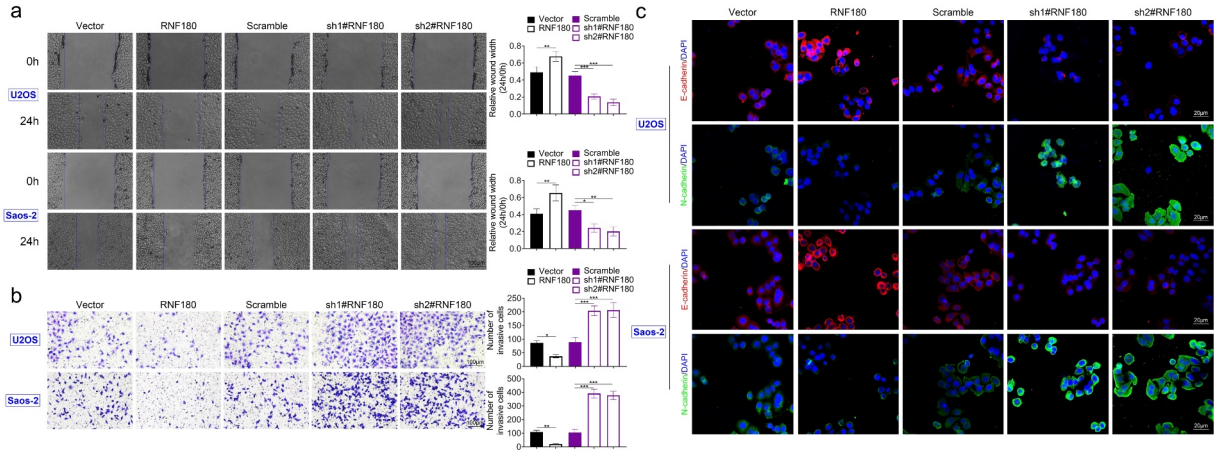

Figure 3. RNF180 inhibits migration and invasion of OS cells. U2OS and Saos-2 cells were transfected with vector, pcDNA3.1-RNF180, scramble shRNA or RNF180 shRnas. (a) Cell migration was evaluated using wound healing assay (bar = 100 μm). (b) Cell invasive ability was assessed by Transwell assay (bar = 100 μm). (c) the expression of E-cadherin and N-cadherin was detected using immunofluorescence assay (bar = 20 μm). RNF180: Ring Finger Protein 180; *p < 0.05, **p < 0.01, ***p < 0.001.

RNF180 inhibits proliferation, migration and invasion in OS cells by targeting CBX4

The U2OS cell viability was significantly inhibited after RNF180 over-expression yet the effect was reversed by further CBX4 over-expression (Figure 6a). Similarly, the cell colony number was decreased in RNF180 group yet increased in CBX4 group, and both effects were partially abolished after RNF180-CBX4 co-transfection (Figure 6b,c). Tunel positive cells were significantly increased after RNF180 over-expression yet decreased in CBX4 over-expression, and the effects were abrogated after RNF180-CBX4 co-transfection (Figure 6d,e). The migration and invasion were inhibited by RNF180 over-expression yet the effects were relieved after RNF180-CBX4 co-transfection (Figure 6f,g,h and i). The protein level of E-cadherin was elevated by RNF180 yet reduced by CBX4, which were partially abolished in RNF180+CBX4 group. The N-cadherin protein level showed the opposite trends (Figure 6j). All these results suggested that RNF180 suppresses proliferation, migration, and invasion in OS cells through down-regulating CBX4.

RNF180 inhibits OS tumor growth in mouse model

We established an OS xenograft model in mice. Tumor volume and tumor weight were significantly smaller after RNF180 over-expression (Figure 7a,b and c). IHC assay showed that the protein levels of RNF180 and E-cadherin were remarkably elevated after RNF180 over-expression, yet the Ki67 and CBX4 protein levels were decreased (Figure 7d). *In vivo* imaging showed that fluorescence signal was observed in lung, limbs, and head after OS cell implantation in vector group, yet signal was weaker in RNF180 group, and mainly observed in lung and head (Figure 7e). Further H&E staining showed that lung metastatic nodes were obviously less in RNF180 group compared with control (Figure 7f). These results indicated that RNF180 inhibits tumor growth and metastasis in OS.

Discussion

The enigmatic molecular mechanism of OS development contributes to its unsatisfactory prognosis. Here we investigated the influences of RNF180 on OS progression. The role of RNF180 is well demonstrated in gastric cancer according to previous researches. RNF180 is significantly down-regulated in gastric cancer owing to the hyper-methylation of CpG sites in RNF180 promoter, which further predicts poor survival of gastric cancer patients [22,23]. RNF180 knockdown by *in vitro* methylation promotes cell growth and suppresses cell apoptosis in gastric cancer, lower RNF180 expression is also correlated with advanced TNM stages and cisplatin resistance,

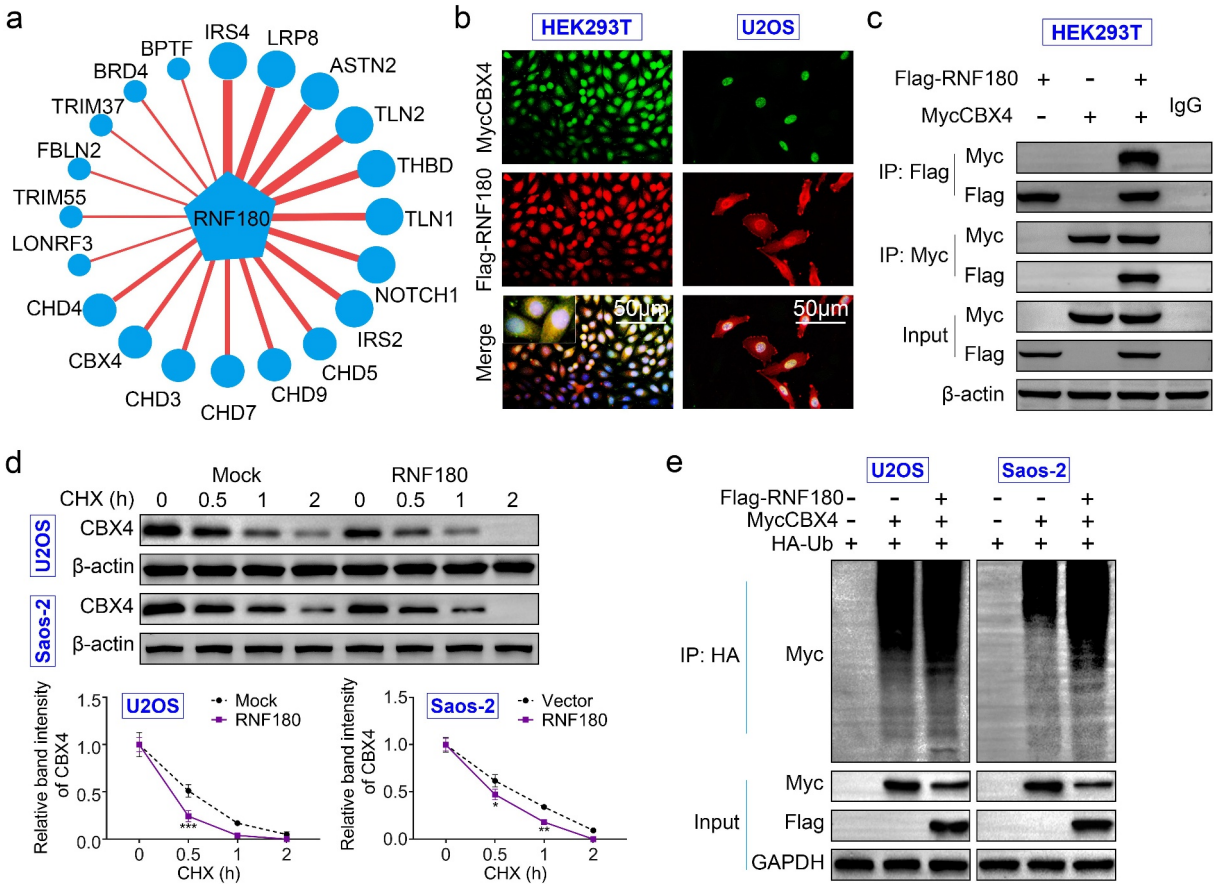

Figure 4. RNF180 is a putative E3 ubiquitin enzyme of CBX4. (a) the predicted targets of RNF180 on Ubibrowser database were shown. (b) the localization of RNF180 and CBX4 in HEK293T and U2OS cells was observed using immunofluorescence assay (bar = 50 μm). (c) U2OS and Saos-2 cells were transfected with Flag-RNF180, Myc-CBX4 or Flag-RNF180+Myc-CBX4. The interaction between RNF180 and CBX4 was detected using co-immunoprecipitation. (d) Cells were transfected with vector or pcDNA3.1-RNF180. The protein levels of CBX4 after CHX treatment was detected using western blotting. (e) the ubiquitylation of CBX4 was evaluated by immunoprecipitation using HA tag, followed by western blotting detection. RNF180: Ring Finger Protein 180; CBX4: chromobox homolog 4; CHX: cycloheximide. *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$.

indicating that RNF180 acts as a tumor suppressor in gastric cancer [8,24]. RNF180 also acts as an independent predictor for survival of non-small cell lung cancer patients [9]. In this study, we first found that RNF180 was significantly down-regulated in OS tissues at both mRNA and protein levels, which was consistent with TCGA database [12]. RNF180 was methylated in both OS tissues and cell lines detected by MSP, indicating that hypermethylation of RNF180 contributed to its weak expression in OS. We further explored the impacts of RNF180 on OS developments using both gain of function and loss of function assays. RNF180 overexpression in OS cell lines inhibited cell viability and proliferation yet promoted cell apoptosis. RNF180 silencing by shRNA, on the contrary, promoted cell proliferation yet suppressed apoptosis, indicating that RNF180 inhibits cell growth in OS. Besides, RNF180 over-expression inhibited migration, invasion, and epithelial-mesenchymal transition (EMT), while RNF180 knockdown showed the opposite effects. We also validated the anti-tumor effect of RNF180 using *in vivo* assays. We established a xenograft tumor model by injecting OS cells, and smaller tumor size and lower tumor weight were observed in RNF180 over-expression group. Lower Ki67 level and higher E-cadherin level were observed after RNF180 over-expression in resected tumor tissues. In addition, lung metastasis account for over 90% of death is OS patients without polychemotherapy [1,25]. In this study, severe metastasis to head, limbs, and especially lung was observed in OS mouse model. However, only moderate

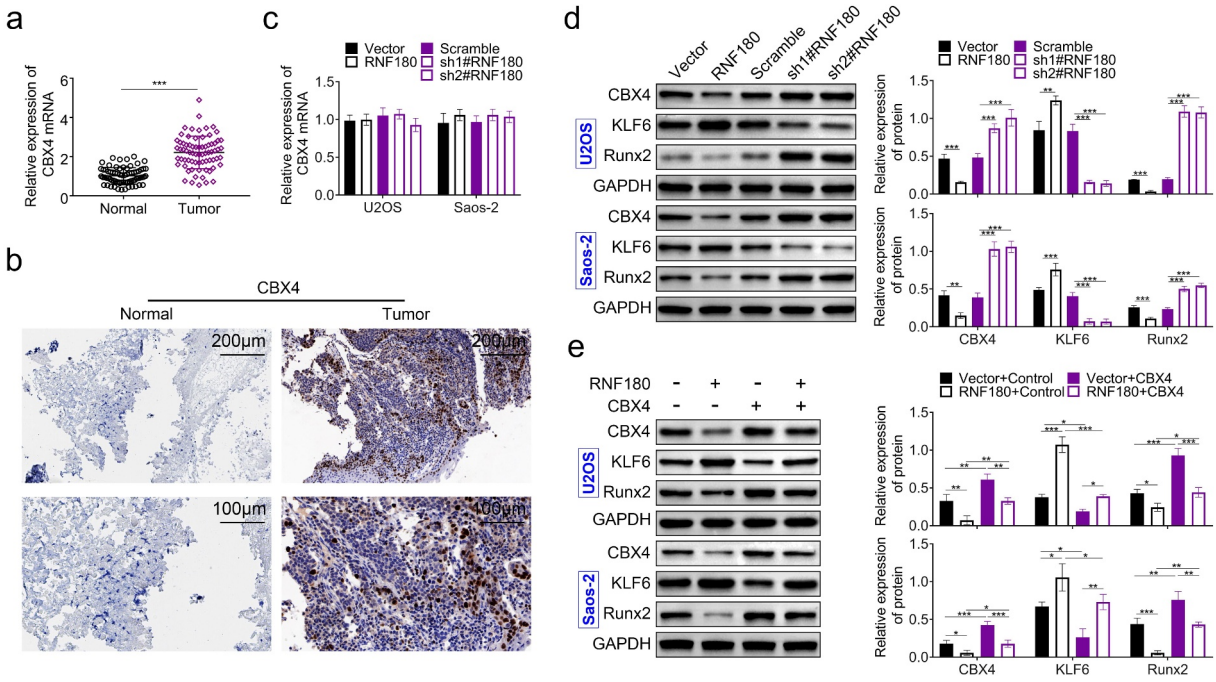

Figure 5. RNF180 regulates KLF6 and Runx2 expression through targeting CBX4. (a) the mRNA level of CBX4 in OS and adjacent normal tissues were measured using quantitative real-time PCR ($n = 72$). (b) the correlation between RNF180 level and CBX4 level in OS tissues were analyzed. (b) the protein level of CBX4 in OS and adjacent normal tissues was evaluated using immunohistochemistry (bar = 200 μm, 100 μm). (c) Relative mRNA level of CBX4 in both U2OS and Saoa-2 cells transfected with vector, pcDNA3.1-RNF180, scramble shRNA, or RNF180 shRNA. (d, e) U2OS and Saos-2 cells were transfected with vector, pcDNA3.1-RNF180, scramble shRNA, RNF180 shRnas, vector + control, pcDNA3.1-RNF180 + control, vector + pcDNA3.1-CBX4, pcDNA3.1-RNF180 + pcDNA3.1-CBX4. Protein levels of CBX4, KLF6 and Runx2 were detected by western blotting. OS: osteosarcoma; RNF180: Ring Finger Protein 180; CBX4: chromobox homolog 4; KLF6: Kruppel like factor 6; Runx2: RUNX family transcription factor 2; *p < 0.05, **p < 0.01, ***p < 0.001.

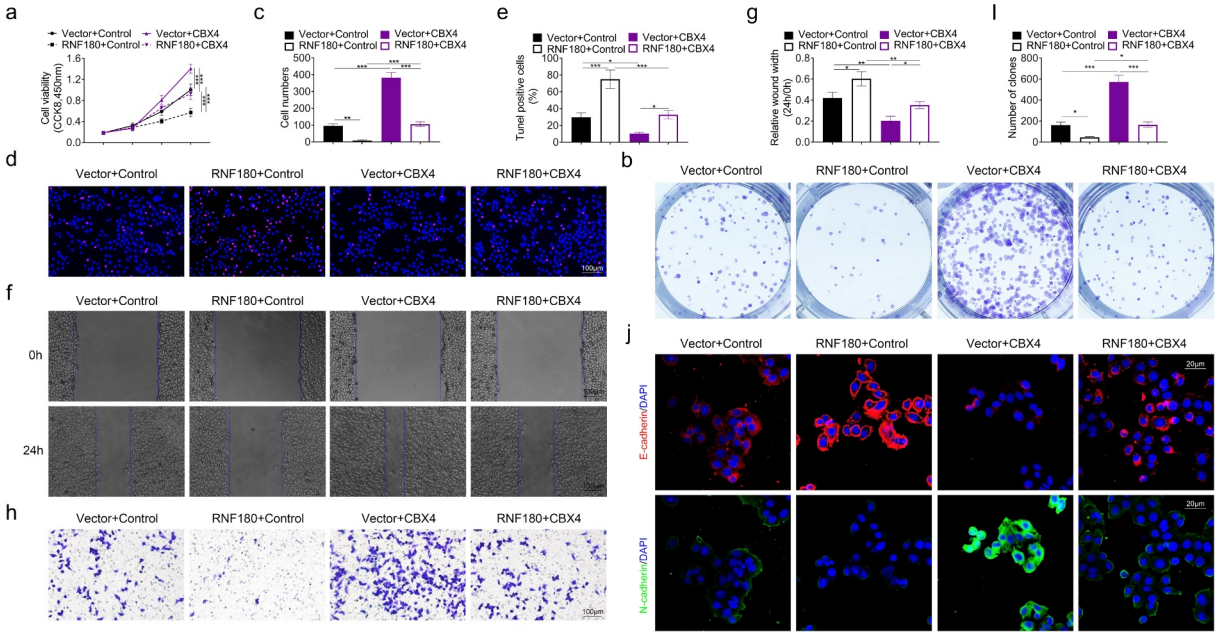

Figure 6. RNF180 inhibits proliferation, migration and invasion in OS cells by targeting CBX4. U2OS and Saos-2 cells were transfected with vector + control, pcDNA3.1-RNF180 + control, vector + pcDNA3.1-CBX4, pcDNA3.1-RNF180 + pcDNA3.1-CBX4. (a) Cell viability was measured using cell counting kit-8 assay. (b, c) Cell proliferation was evaluated using clone forming assay. (d, e) Tunel assay was carried out to assess cell apoptosis (bar = 100 μm). (f, g) Wound healing was performed to evaluate cell migration (bar = 100 μm). (h, i) Transwell was performed to assess cell invasion (bar = 100 μm). (j) the protein levels of E-cadherin and N-cadherin were detected using immunofluorescence assay (bar = 20 μm). OS: osteosarcoma; RNF180: Ring Finger Protein 180; CBX4: chromobox homolog 4; Tunel: TdT-mediated dUTP Nick-End Labeling; *p < 0.05, **p < 0.01, ***p < 0.001.

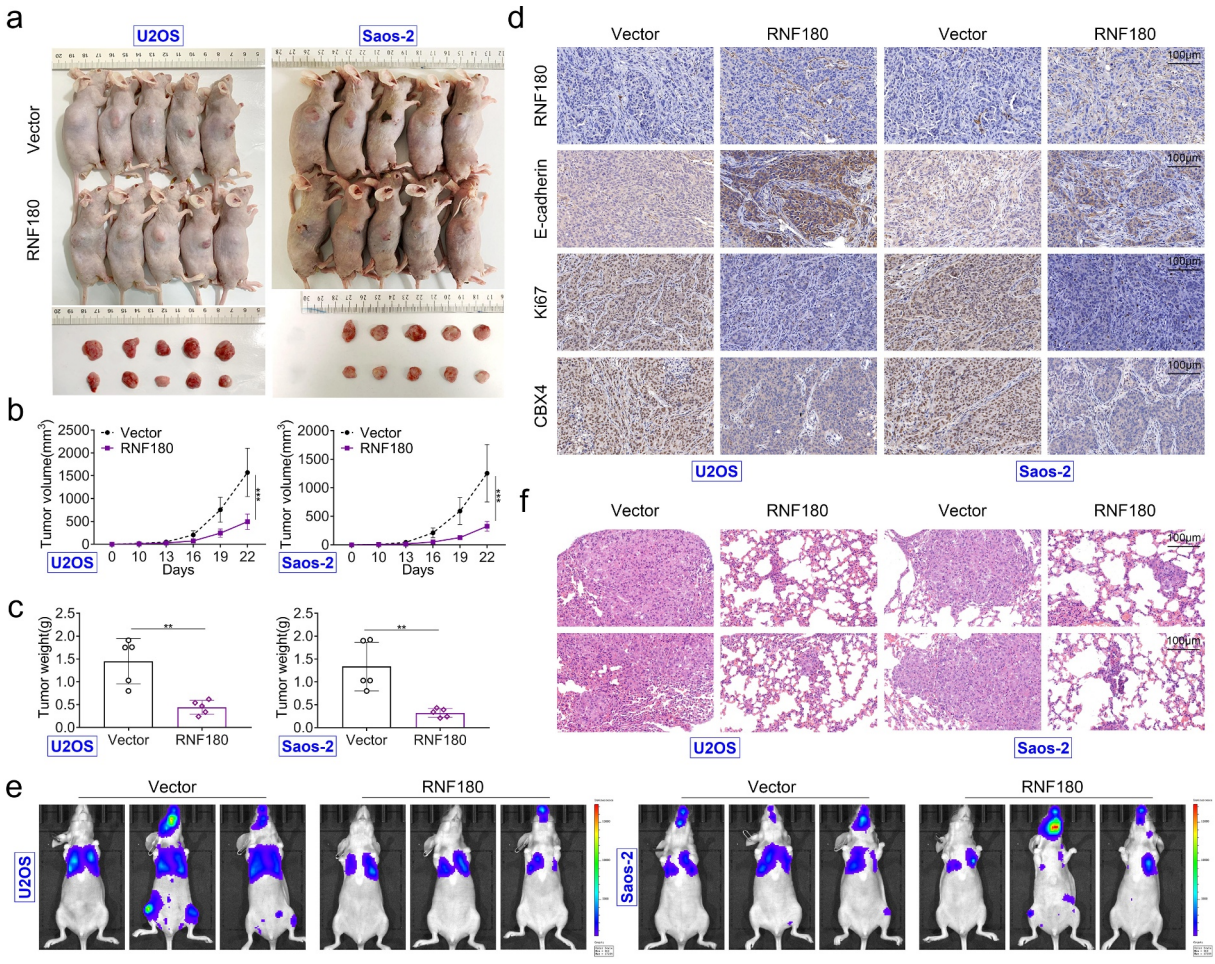

Figure 7. RNF180 inhibits OS tumor growth in mouse model. Balb/c node mice were injected with U2OS or Saos-2 cells via tail vein. Cells were transfected with vector or EGFP tagged pcDNA3.1-RNF180. (a) Xenograft tumors were shown ($n = 5$). (b, c) Tumor volume and tumor weight were measured and analyzed 22 days after implantation. (d) Immunohistochemistry was performed to evaluate protein levels of RNF180, E-cadherin, Ki67 and CBX4 (bar = 100 μm). (e) Tumor metastasis was shown by in vivo imaging (bar = 100 μm). (f) Lung metastasis nodes was observed after hematoxylin-eosin staining. OS: osteosarcoma; RNF180: Ring Finger Protein 180; *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$.

metastasis to lung and head was observed after RNF180 over-expression. Lung metastasis nodules in RNF180 group were also obviously less than that in control group. All these results demonstrated that RNF180 inhibits proliferation, migration, invasion, EMT, and lung metastasis in OS, and RNF180 might act as a tumor-suppressor and potential therapeutic target in OS treatment.

RNF180 also acts as an important E3 ubiquitin ligase via regulating protein degradation in cancer. For instance, RNF180 regulates the expression of Ras homolog gene family member C via ubiquitination and further protein degradation through proteasomal pathway in gastric cancer [26]. RNF180 restores the expression of ADAMTS9 via ubiquitination of DNA methyltransferase 3α (DNMT3A) in gastric cancer [27]. Here we validated CBX4 as a novel substrate of RNF180 in OS. RNF180 and CBX4 were co-localized mainly in nucleus, and their interaction was validated using Co-IP. RNF180 accelerated the decline of CBX4 level after treatment of CHX, a protein synthesis inhibitor. RNF180 also elevated the ubiquitination level of CBX4 in OS cells, indicating that RNF180 reduces CBX4 expression in OS via ubiquitination and further proteasomal pathway. CBX4 acts as an oncogene in various cancers including OS, breast cancer, lung cancer, and hepatocellular carcinoma [13,16,28,29]. Of note, CBX4 is over-expressed in OS cell lines and tissues [16]. CBX4 promotes metastasis via interacting with casein kinase 1α and up-regulating Runx2 [16]. CBX4 also regulates the malignant behavior of OS through

interacting with metabolic glutamate receptor 4 (GRM4). In the present study, CBX4 is significantly up-regulated in OS tissues at both mRNA and protein levels. Nevertheless, RNF180 exerted regulatory effects on CBX4 at the protein level rather than mRNA in OA cell lines. Besides, RNF180 further up-regulated KLF6 yet inhibited Runx2 expression, both of which are downstream targets of CBX4. KLF6 acts as a tumor suppressor yet Runx2 are related to metastasis and resistance to chemotherapy in OS [16,18,19,30,31]. In addition, RNF180 inhibited proliferation, migration, invasion, and EMT in OS, which was partially reversed by CBX4 overexpression.

In summary, RNF180 is down-regulated in OS due to promoter hypermethylation, and RNF180 suppresses OS progression through regulating ubiquitination of CBX4. Our findings indicated that the RNF180-CBX4 axis might be a promising target for diagnosis and therapeutic of OS.

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Data availability materials

All data generated or analyzed during this study are included in this published article. The datasets used and/or analyzed during the present study are available from the corresponding author on reasonable request.

Ethics approval

All procedures performed in studies involving human participants were in accordance with the standards upheld by the Ethics Committee of the First Clinical Hospital affiliated to Harbin Medical University and with those of the 1964 Helsinki Declaration and its later amendments for ethical research involving human subjects. All animal experiments were approved by the Ethics Committee of the First Clinical Hospital affiliated to Harbin Medical University for the use of animals and conducted in accordance with the National Institutes of Health Laboratory Animal Care and Use Guidelines.

Statement of informed consent

Written informed consent was obtained from a legally authorized representative(s) for anonymized patient information to be published in this article.

Contribution of authors

Qirui Zhao and Ning Liu designed the study, supervised the data collection, Tongtong Xu analyzed the data, interpreted the data, Keguan Song prepare the manuscript for publication and reviewed the draft of the manuscript. All authors have read and approved the manuscript.

References

[1] Ritter J, Bielack S. S. Osteosarcoma. Ann Oncol. 2010;21(*Suppl* 7):vii320–5.
[2] Dahlin DC. Pathology of osteosarcoma. Clin Orthop Relat Res. 1975 (111):23–32.
[3] Ando K, Heymann MF, Stresing V, et al. Current therapeutic strategies and novel approaches in osteosarcoma. Cancers (Basel). 2013;5(2):591–616.
[4] Wagle S, Park SH, Kim KM, et al. DBC1/CCAR2 is involved in the stabilization of androgen receptor and the progression of osteosarcoma. Sci Rep. 2015;5:13144.
[5] Ogawa M, Mizugishi K, Ishiguro A, et al. Rines/RNF180, a novel RING finger gene-encoded product, is a membrane-bound ubiquitin ligase. Genes to Cells. 2008;13(4):397–409.
[6] Joazeiro CA, Weissman AM. RING finger proteins: mediators of ubiquitin ligase activity. Cell. 2000;102 (5):549–552.
[7] Pickart CM. Mechanisms underlying ubiquitination. Annu Revi Biochem. 2001;70:503–533.
[8] Cheung KF, Lam CN, Wu K, et al. Characterization of the gene structure, functional significance, and clinical application of RNF180, a novel gene in gastric cancer. Cancer. 2012;118(4):947–959.
[9] Liu H, Yang P, Li X, et al. Ring finger protein 180 is associated with biological behavior and prognosis in patients with non-small cell lung cancer. Oncol Lett. 2020;20(4):35.
[10] Tu M, He L, You Y, et al. EFTUD2 maintains the survival of tumor cells and promotes hepatocellular carcinoma progression via the activation of STAT3. Cell Death Amp Dis. 2020;11(10):1–12.
[11] Deng J, Liang H, Zhang R, et al. Clinical and experimental role of ring finger protein 180 on lymph node metastasis and survival in gastric cancer. Br J Surg. 2016;103(4):407–416.
[12] Chandrashekar DS, Bashel B, Balasubramanya SAH, et al. UALCAN: a portal for facilitating tumor subgroup gene expression and survival analyses. Neoplasia. 2017;19(8):649–658.

[13] Li J, Xu Y, Long XD, et al. Cbx4 governs HIF-1alpha to potentiate angiogenesis of hepatocellular carcinoma by its SUMO E3 ligase activity. Cancer Cell. 2014;25(1):118–131.
[14] Wang B, Tang J, Liao D, et al. Chromobox homolog 4 is correlated with prognosis and tumor cell growth in hepatocellular carcinoma. Ann Surg Oncol. 2013;20 (*Suppl* 3):S684–92.
[15] Jiang N, Niu G, Pan YH, et al. CBX4 transcriptionally suppresses KLF6 via interaction with HDAC1 to exert oncogenic activities in clear cell renal cell carcinoma. EBioMedicine. 2020;53:102692.
[16] Wang X, Qin G, Liang X, et al. Targeting the CK1α/CBX4 axis for metastasis in osteosarcoma. Nat Commun. 2020;11(1):1141.
[17] Yang J, Cheng D, Zhu B, et al. Chromobox homolog 4 is positively correlated to tumor growth, survival and activation of HIF-1alpha signaling in human osteosarcoma under normoxic condition. J Cancer. 2016;7 (4):427–435.
[18] Chen K, Chen Y, Zhu XD, et al. Expression and significance of Kruppel-like factor 6 gene in osteosarcoma. Int Orthop. 2012;36(10):2107–2111.
[19] Jianwei Z, Enzhong B, Fan L, et al. Effects of Kruppel-like factor 6 on osteosarcoma cell biological behavior. Tumour Biol. 2013;34(2):1097–1105.
[20] Martin JW, Zielenska M, Stein GS, et al. The role of RUNX2 in osteosarcoma oncogenesis. Sarcoma. 2011;2011:282745.
[21] Vega OA, Lucero CMJ, Araya HF, et al. Wnt/beta-catenin signaling activates expression of the bone-related transcription factor RUNX2 in select human osteosarcoma cell types. J Cell Biochem. 2017;118(11):3662–3674.
[22] Deng J, Liang H, Ying G, et al. Methylation of CpG sites in RNF180 DNA promoter prediction poor survival of gastric cancer. Oncotarget. 2014;5 (10):3173–3183.
[23] Han F, Liu S, Jing J, et al. Identification of high-frequency methylation sites in RNF180 promoter region affecting expression and their relationship with prognosis of gastric cancer. Cancer Manage Res. 2020;12:3389–3399.
[24] Wang H, Lu Y, Wang M, et al. Correction to: roles of E3 ubiquitin ligases in gastric cancer carcinogenesis and their effects on cisplatin resistance. J Mol Med (Berl). 2021;99:1021–1022.
[25] Doyle LA. Sarcoma classification: an update based on the 2013 World Health Organization classification of tumors of soft tissue and bone. Cancer. 2014;120 (12):1763–1774.
[26] Wu Z, Liu H, Sun W, et al. RNF180 mediates STAT3 activity by regulating the expression of RhoC via the proteasomal pathway in gastric cancer cells. Cell Death Amp Dis. 2020;11(10):881.
[27] Sun W, Ma G, Zhang L, et al. DNMT3A-mediated silence in ADAMTS9 expression is restored by RNF180 to inhibit viability and motility in gastric cancer cells. Cell Death Amp Dis. 2021;12(5):428.
[28] Zeng JS, Zhang ZD, Pei L, et al. CBX4 exhibits oncogenic activities in breast cancer via notch1 signaling. Int J Biochem Cell Biol. 2018;95:1–8.
[29] Hu C, Zhang Q, Tang Q, et al. CBX4 promotes the proliferation and metastasis via regulating BMI-1 in lung cancer. J Cell Mol Med. 2020;24(1):618–631.
[30] Hsu LS, Huang RH, Lai HW, et al. KLF6 inhibited oral cancer migration and invasion via downregulation of mesenchymal markers and inhibition of MMP-9 activities. Int J Med Sci. 2017;14(6):530–535.
[31] Li N, Luo D, Hu X, et al. RUNX2 and osteosarcoma. Anticancer Agents Med Chem. 2015;15(7):881–887.