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Interfering with UBE2L3 expression targets regulation of MLKL to promote necroptosis inhibition of growth in osteosarcoma

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Abstract

Background In previous studies, elevated expression of UBE2L3 has been observed in osteosarcoma cells, and silencing UBE2L3 has been shown to promote oxidative stress and induce necroptosis. However, the exact molecular mechanisms underlying these findings remain unclear.

Objective The purpose of this study is to investigate the molecular mechanisms by which interfering with UBE2L3 expression promotes necroptosis and impacts the progression of osteosarcoma, building upon previous in vitro cell experiments.

Methods Osteosarcoma cells were transfected with shNC and shUBE2L3 plasmids, and the cells were injected into the right tibia of nude mice to establish a tumor xenograft model. The growth rate, changes in body weight, and tumor volume of the mice in each group were observed. After 15 days, the mice were sacrificed, and the tumors were dissected and analyzed for tumor volume. Immunohistochemical staining was performed to detect changes in the expression of necroptosis-related proteins, such as PCNA, p-MLKL, and p-RIP1. Additionally, U2OS and HOS cells were transfected with UBE2L3-silencing plasmids, and immunoprecipitation was performed to investigate the interaction between UBE2L3 and the necroptosis protein MLKL. By combining these experiments, we aim to evaluate the impact of UBE2L3 on necroptosis both in vitro and in vivo and elucidate its specific role in targeting MLKL to regulate necroptosis as a therapeutic approach for osteosarcoma.

Results After interfering with UBE2L3, the growth rate of tumors in nude mice significantly slowed down, accompanied by a notable reduction in tumor volume and weight. These findings suggest that inhibiting the expression of UBE2L3 can suppress the growth of osteosarcoma. Furthermore, immunohistochemical analysis revealed that following UBE2L3 interference, the intensity of staining for the necrotic proteins p-MLKL and p-RIP1 was increased and PCNA staining was decreased, indicating that interfering with UBE2L3 expression can promote necroptosis. Moreover, through transfection of UBE2L3 silencing plasmids into osteosarcoma cells in vitro, immunoprecipitation and ubiquitination results demonstrated that UBE2L3 can specifically bind to MLKL.

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Overexpression of UBE2L3 promoted the ubiquitination of MLKL and reduced its expression. Thus, down-regulation of UBE2L3 could modulate downstream MLKL expression and promote necrosis of osteosarcoma cells.

Conclusion UBE2L3 selectively binds to MLKL, exerting ubiquitination-mediated regulation on downstream MLKL. Decreased expression of UBE2L3 modulates MLKL expression and promotes necrosis, thereby inhibiting osteosarcoma growth.

Keywords Osteosarcoma, Necroptosis, UBE2L3, MLKL

Introduction

Osteosarcoma is a rare but serious malignant tumor that primarily occurs in the growth areas of bones and soft tissues. According to recent epidemiological data, approximately 1–2 per million people are diagnosed with osteosarcoma worldwide, with a higher prevalence among children and adolescents aged 10–25 years [1]. The incidence rate of osteosarcoma is slightly higher in males than females, with a male-to-female ratio of approximately 1.4:1 [2, 3]. The aggressive nature and high susceptibility to metastasis are among the defining characteristics and major clinical challenges of osteosarcoma. If left untreated, osteosarcoma can cause localized pain, swelling, and pathological fractures due to tumor-induced bone destruction. As the disease progresses, tumor cells primarily metastasize to the lungs through the bloodstream, while lymph node and other bone metastases are less common. This propensity for distant metastasis, particularly to the lungs, contributes to the high lethality of osteosarcoma, with a 5-year survival rate of approximately 20–30% for metastatic disease [4, 5].

The pathogenesis of osteosarcoma is associated with necroptosis, a form of regulated cell death mediated by RIPK1, RIPK3, and MLKL. Necroptosis is distinct from apoptosis, as it involves the loss of cell membrane integrity and the release of cellular contents, often triggering inflammatory responses [6, 7]. Recent studies suggest that osteosarcoma cells may undergo necroptosis under specific conditions when exposed to chemotherapy drugs or radiation therapy, primarily due to the accumulation of reactive oxygen species (ROS) and DNA damage [8, 9]. Dysregulated activation of pathways such as p53 may also indirectly influence necroptosis by modulating oxidative stress responses [10, 11]. Given the involvement of multiple signaling pathways in necroptosis regulation, further exploration of its molecular mechanisms in osteosarcoma may lead to innovative therapeutic strategies, enhancing the efficacy of conventional treatments and improving patient outcomes. MLKL is a key execution protein in the necroptosis pathway, playing a central role in cell death signal transduction. Upon phosphorylation and activation, MLKL translocates to the cell membrane, where it induces membrane rupture and the release of cellular contents, ultimately triggering necroptosis [12, 13]. While MLKL has been shown to play a significant role in the progression of cancers such as ovarian [14] and pancreatic cancer [15], its involvement in OS remains relatively understudied.

In our preliminary research, our team discovered that UBE2L3 expression was abnormally elevated in various osteosarcoma cell lines. Furthermore, transfection with a UBE2L3 silencing plasmid was found to inhibit the proliferation, invasion, migration, and promote apoptosis in U2OS and HOS osteosarcoma cells [13]. Further investigation into the mechanism revealed that UBE2L3 can inhibit oxidative stress levels, thereby inhibiting necroptosis. Therefore, in this research, we focus on uncovering how UBE2L3 facilitates necroptosis through the ubiquitination of MLKL, a key effector in the necroptotic pathway. Additionally, we aim to validate these findings in vivo using osteosarcoma nude mouse models to comprehensively evaluate the role of UBE2L3 in osteosarcoma progression. This study seeks to provide novel mechanistic insights and scientific evidence for targeting UBE2L3 as a potential therapeutic strategy in osteosarcoma treatment.

Materials and methods

Materials

Human osteosarcoma cell lines (U2OS, HOS) were purchased from Wuhan Punoise Life Technology Co., Ltd. DMEM medium, pancreatin, double antibodies, and fetal bovine serum were obtained from GIBCO. SDS-PAGE gel preparation kit and IP cell lysis buffer were purchased from Beijing Solarbio Technology Co., Ltd. Immunoprecipitation kit, GAPDH antibody (Abmart, M20006), UBE2L3 antibody (Abmart, P30053), RIP1 antibody (Abmart, P30045), PCNA antibody (Abmart, P30004), MLKL antibody (Abmart, P30131), p-RIP1 antibody (CST, 65746), and p-MLKL antibody (Abcam, ab196436) were purchased from the respective manufacturers. UBE2L3 silencing plasmid was obtained from Guangzhou Hanheng Company.

Cell culture and transfection

The well-growing human osteosarcoma cell lines U2OS and HOS were cultured in pH 7.4 DMEM medium (containing 10% fetal bovine serum, 100 U/L penicillin, and 100 mg/L streptomycin) and placed in a 37 °C, 5% CO_2 humidified incubator. The cells were cultured in DMEM

medium (containing 10% fetal bovine serum) and passaged after reaching confluence. After obtaining a sufficient number of passage cells, they were seeded in culture plates for experimental grouping. According to the transfection reagent instructions, UBE2L3 silencing plasmid 1, UBE2L3 plasmid 2, and negative control adenovirus were transfected into U2OS and HOS cells.

Establishment of osteosarcoma nude mice model

The skin at the right hind limb injection site of nude mice was disinfected with 75% alcohol for 3 times. A 1 mL syringe was used for tumor cell injection. Same cell quantities ($1 \times 10\hat{}6$/200μL) were injected into the right tibia of the nude mice. During the injection process, caution was taken to avoid cell suspension leakage caused by the movement of the mice, which could affect the modeling effect. Successful modeling was confirmed when tumors appeared in the right hind limbs of the nude mice. The animal groups included sh-NC group, sh-UBE2L3-1 group, and sh-UBE2L3-2 group.

Measurement of nude mouse weight and tumor volume

Tumor volume measurement: The tumor volume was measured on the, 3rd, 5th, 7th, 10th, and 15th day of gavage administration. The measurement method involved restraining the nude mouse on the operating table, fully extending the right hind limb of the mouse, and using a vernier caliper to measure the long and short diameters of the tumor. The data was recorded and the tumor volume was calculated using the formula: Tumor volume = (tumor long diameter × tumor short diameter^2)/2. After 15 days, the nude mice was euthanized with an overdose of sodium pentobarbital. The tumor tissue in the right hind limb of the nude mouse was dissected by tissue scissors, and care was taken to maintain tumor integrity. The weight of the tumor tissue was recorded. The removed tumor tissue was used for subsequent immunohistochemical experiments.

Immunohistochemical detection of necroptosis protein expression

The tumor tissue was isolated from the nude mice and the surrounding tissue was removed. Subsequently, the tumor tissue was fixed in 4% paraformaldehyde for 24 h, followed by embedding, sectioning, dewaxing, and rehydration. When processing tissue sections, the first step was antigen retrieval and blocking. Then, rabbit monoclonal antibodies against p-RIP1, PCNA, and p-MLKL were applied to the sections. The incubation process lasted for 1 h at 37 °C. Next, a secondary antibody labeled with horseradish peroxidase (HRP) was introduced, and this step continued for 30 min at room temperature. After completing these steps, the sections were stained with the DAB reagent and lightly counterstained with hematoxylin. Subsequently, the sections underwent dehydration, clearing, and mounting. After this series of procedures, the sections were observed and recorded under a microscope.

Immunoprecipitation

U2OS and HOS cells were washed three times with ice-cold PBSand lysed using RIPA buffer (Beyotime, P0013B) supplemented with MG132 (Roche, 04693132001) and DMSO (Roche, 04906837001). After centrifugation at 12,000 × g for 15 min at 4 °C, the supernatant was collected, and protein concentrations were determined using a BCA protein quantification kit (Thermo Fisher, 23225) to ensure equal loading. For co-immunoprecipitation (Co-IP), equal amounts of protein were incubated overnight at 4 °C with UBE2L3 antibody (Proteintech, 13641-1-AP) or MLKL antibody (Abcam, ab184718) prebound to Protein A/G beads (Santa Cruz, sc-2003). The immune complexes were washed extensively with PBS, and bound proteins were eluted by boiling the beads in SDS-PAGE loading buffer (Beyotime, P0015L). The samples were separated via SDS-PAGE, transferred onto PVDF membranes (Millipore, ISEQ00010), and analyzed by immunoblotting with antibodies against UBE2L3 and MLKL to detect protein interactions.

Statistical analysis

Each experiment was repeated three times, and the data was analyzed using GraphPad Prism 8.0.1 and SPSS 25.0 software. The results were expressed as mean ± standard deviation (mean ± SD). Differences between multiple groups were compared using ANOVA, while differences between two groups were compared using the t-test. A p-value of less than 0.05 was considered statistically significant.

Results

Inhibition of tumor growth in nude mice with osteosarcoma by UBE2L3 interference

To observe the effect of UBE2L3 interference on the tumor volume of osteosarcoma in nude mice, the tumor volume was measured and calculated on the 3rd, 5th, 7th, 10th, and 15th day after intervention. Starting from the 5th day of intervention, the tumor volumes in the sh-UBE2L3-1 and sh-UBE2L3-2 groups showed a significant decrease compared to the sh-NC group. On the 15th day after intervention, the difference in tumor volume between the sh-UBE2L3-1 and sh-UBE2L3-2 groups and the sh-NC group was statistically significant ($P < 0.05$, Fig. 1A and B). Additionally, the tumor weights in the sh-UBE2L3-1 and sh-UBE2L3-2 groups were significantly lower than the sh-NC group ($P < 0.05$, Fig. 1C). These results indicate that UBE2L3 interference inhibits tumor growth in nude mice with osteosarcoma.

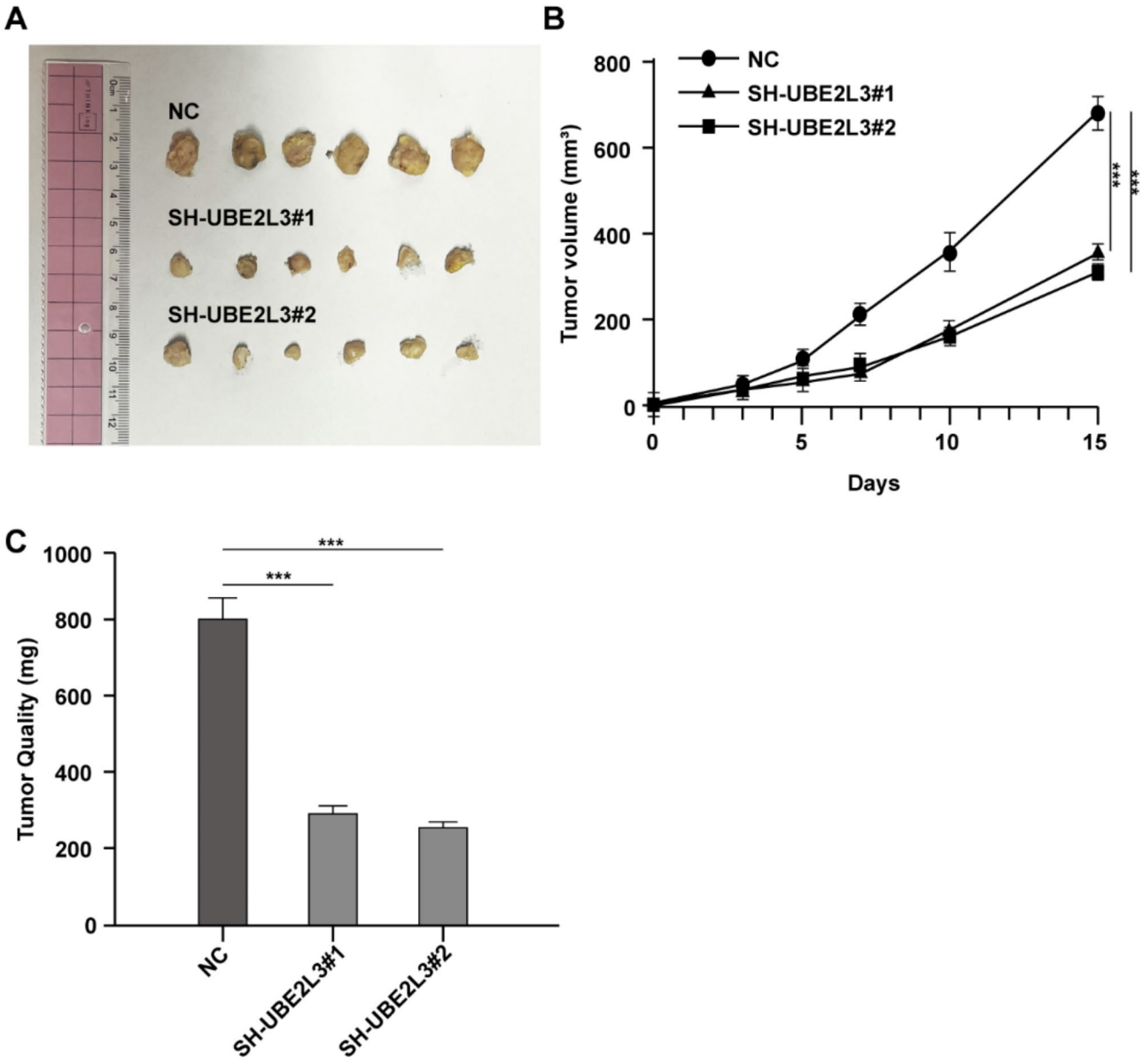

Fig. 1 UBE2L3 knockdown inhibits tumor growth in vivo. (**A**) Representative images of tumors excised from mice injected with control (sh-NC) or UBE2L3 knockdown (sh-UBE2L3#1, sh-UBE2L3#2) cells. (**B**) Tumor volume was measured over 15 days. Tumors in the UBE2L3 knockdown groups grew significantly slower compared to the control group. (**C**) Tumor weight at the endpoint. Tumors in the UBE2L3 knockdown groups were significantly lighter than those in the control group. Data are shown as mean ± SD. ***$P < 0.001$

Inhibition of necroptosis protein expression by UBE2L3 interference

IHC analysis revealed that UBE2L3 knockdown significantly altered the expression levels of PCNA, p-MLKL, and p-RIPK1 in tumor tissues. The PCNA-positive area, which reflects cell proliferation, was markedly higher in the NC group, while it was significantly reduced in the UBE2L3 knockdown groups, suggesting that UBE2L3 knockdown inhibits tumor cell proliferation (Fig. 2A). In contrast, the expression levels of p-MLKL and p-RIPK1, which are critical markers of necroptosis, were significantly elevated in the UBE2L3 knockdown groups compared to the NC group (Fig. 2B and C).

Western blot results showed that UBE2L3 knockdown significantly affected the expression levels of p-RIPK1, RIPK1, p-MLKL, and MLKL (Fig. 3A). In the NC group, the expression levels of p-RIPK1 and p-MLKL were relatively low, whereas they were significantly elevated in the UBE2L3 knockdown groups. In addition, the protein levels of RIPK1 and MLKL were significantly reduced in the UBE2L3 knockdown groups compared to the NC group, with statistically significant differences. Further quantitative analysis revealed that the ratios of p-RIPK1 to RIPK1

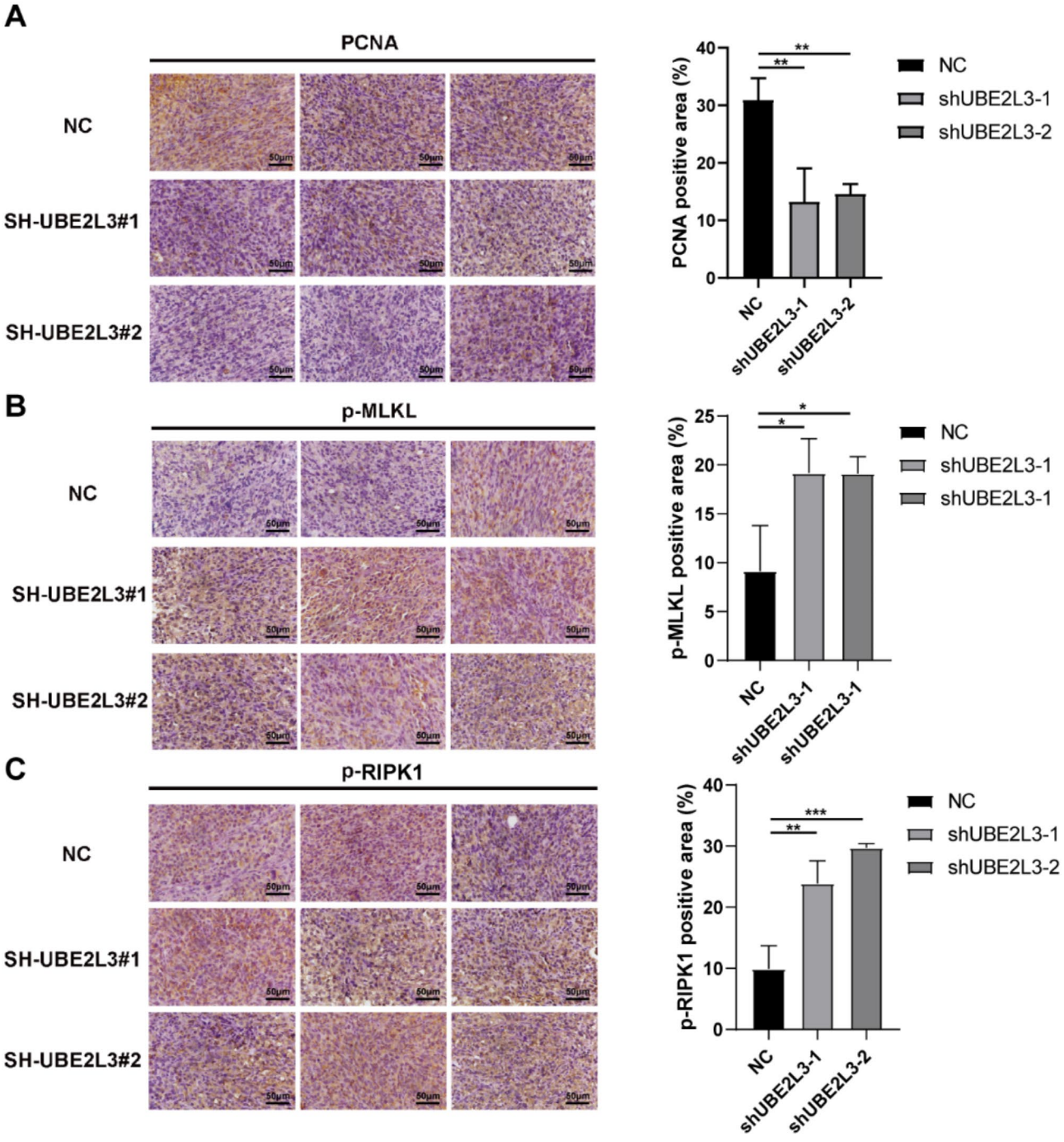

Fig. 2 UBE2L3 knockdown affects cell proliferation and necroptosis markers in tumor tissues. (**A**) Representative IHC staining of PCNA and quantification of PCNA-positive areas in NC and UBE2L3 knockdown groups. (**B**) Representative IHC staining of p-MLKL and quantification of p-MLKL-positive areas. (**C**) Representative IHC staining of p-RIPK1 and quantification of p-RIPK1-positive areas. Data are presented as mean ± SD. *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$

and p-MLKL to MLKL were significantly increased in the UBE2L3 knockdown groups (Fig. 3B).

This indicates that UBE2L3 knockdown may promote necroptosis through activation of the RIPK1/RIPK3/MLKL signaling pathway. Together, these results suggest that UBE2L3 knockdown not only suppresses tumor cell proliferation but also induces necroptosis, providing new insights into the role of UBE2L3 in tumor progression.

Ubiquitination of MLKL protein by UBE2L3

To investigate the regulatory effect of UBE2L3 on MLKL, we conducted a series of experiments in U2OS and HOS cells. Western blot analysis (Fig. 4) showed

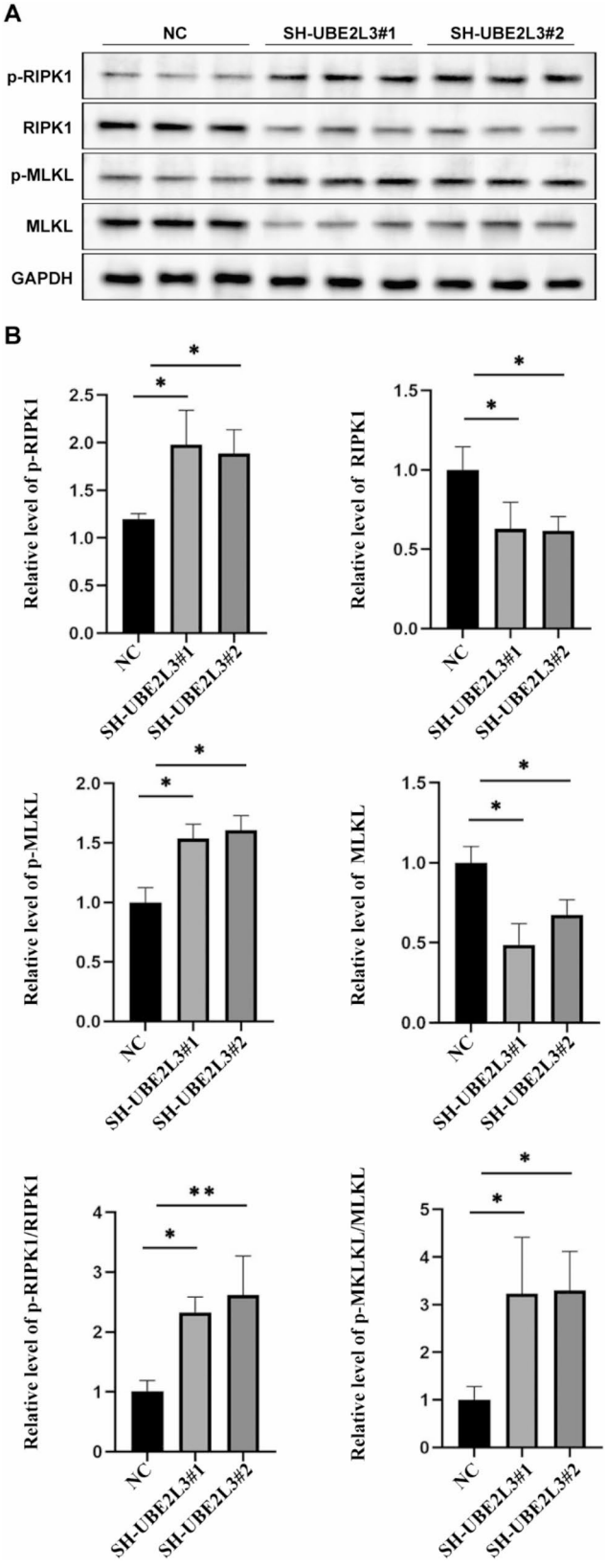

Fig. 3 UBE2L3 knockdown activates the necroptosis signaling pathway. (**A**) Western blot analysis of p-RIPK1, RIPK1, p-MLKL, and MLKL protein levels in NC and UBE2L3 knockdown groups. GAPDH served as the loading control. (**B**) Quantification of protein expression levels normalized to GAPDH, including the ratios of p-RIPK1/RIPK1 and p-MLKL/MLKL. Data are presented as mean ± SD. *$P < 0.05$, **$P < 0.01$

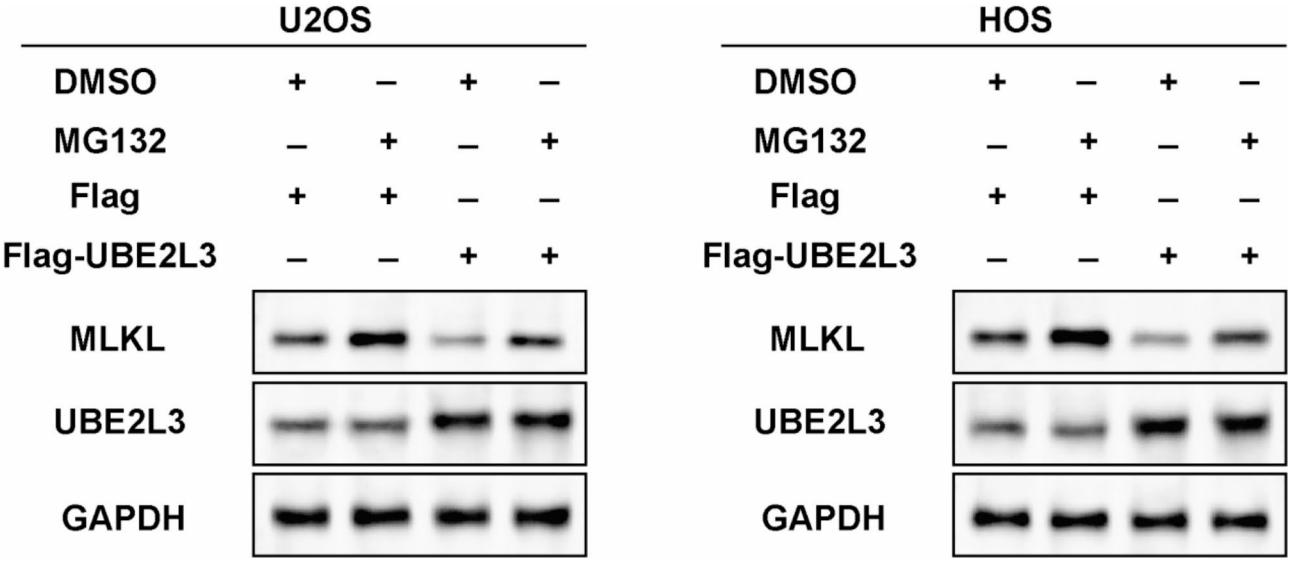

Fig. 4 Western blot validation of the binding between UBE2L3 and MLKL

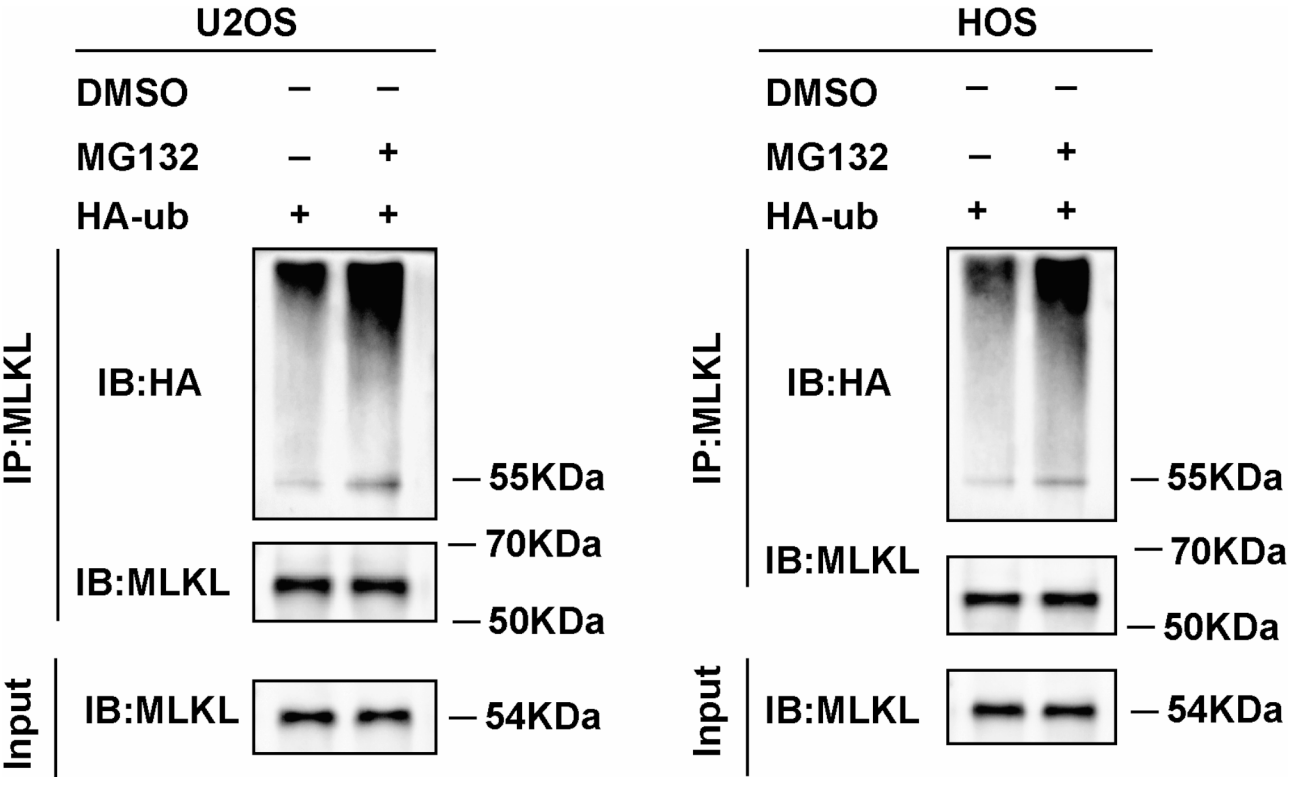

Fig. 5 Autoubiquitination modification of MLKL

that overexpression of UBE2L3 (Flag-UBE2L3) significantly reduced the protein expression level of MLKL, while MLKL levels remained higher in the control group (Flag). Notably, treatment with the proteasome inhibitor MG132 effectively restored MLKL protein levels, suggesting that UBE2L3 promotes MLKL degradation via the proteasome pathway. To confirm the involvement of ubiquitination, we performed co-immunoprecipitation assays using HA-ubiquitin to assess the ubiquitination level of MLKL. Results demonstrated that in the presence of MG132, the ubiquitination level of MLKL significantly increased (Fig. 5), indicating that MLKL ubiquitination is closely associated with proteasomal degradation. To further validate the direct role of UBE2L3 in MLKL

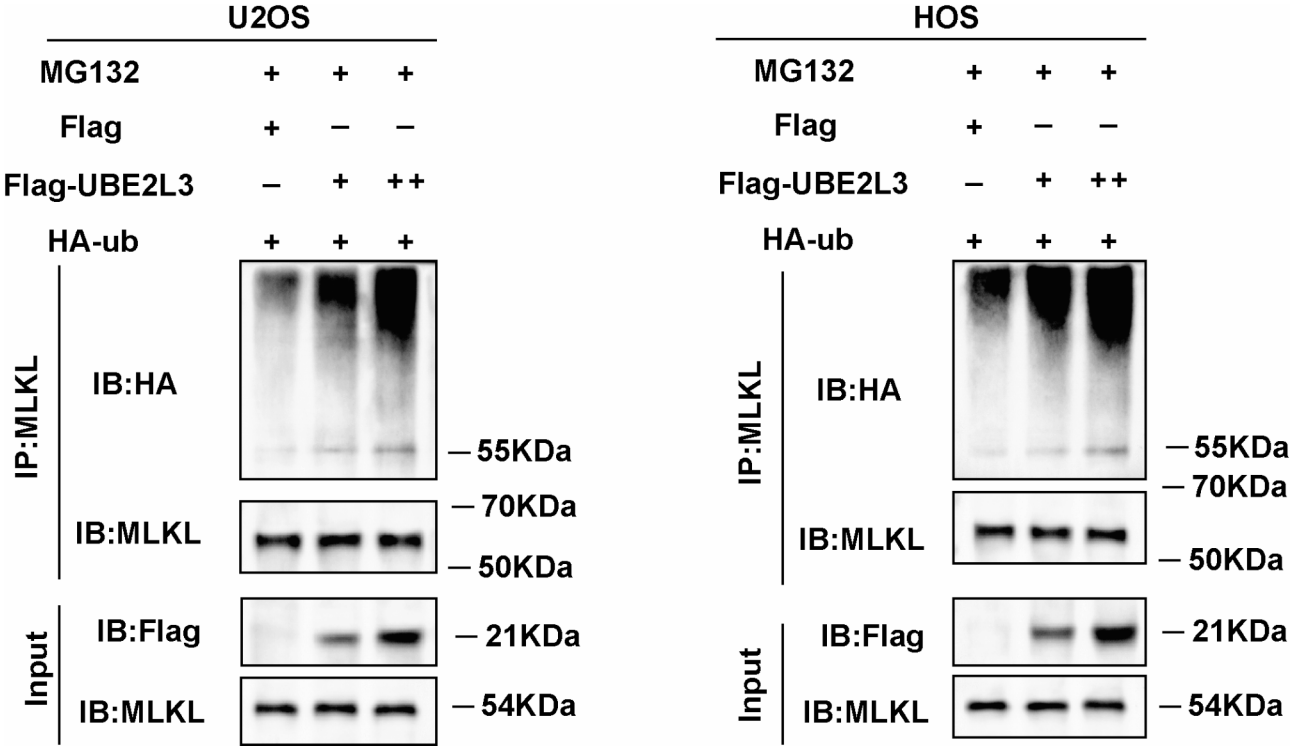

Fig. 6 UBE2L3 promotes ubiquitination modification of MLKL

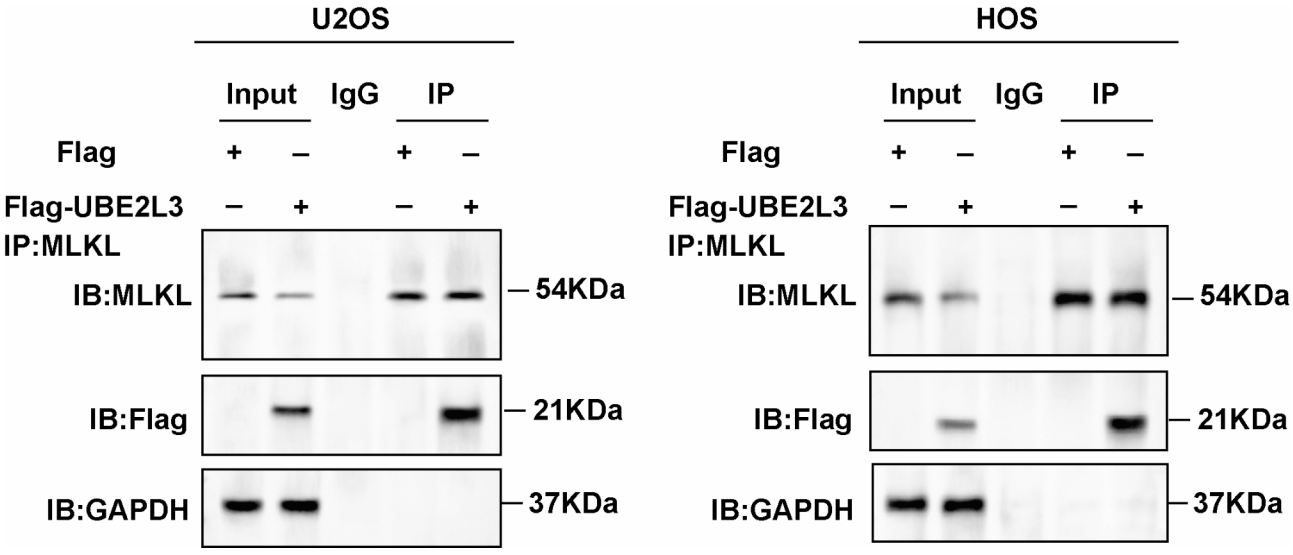

Fig. 7 Immunoprecipitation confirms the interaction between UBE2L3 and MLKL

ubiquitination, we overexpressed UBE2L3 (Flag-UBE2L3) under MG132 treatment. The results showed that increasing UBE2L3 expression markedly enhanced MLKL ubiquitination levels, as evidenced by the elevated HA-ubiquitin signal (Fig. 6). This clearly demonstrates that UBE2L3 mediates MLKL degradation by promoting its ubiquitination. Additionally, co-immunoprecipitation experiments revealed a direct interaction between UBE2L3 and MLKL. In the Flag-UBE2L3 overexpression group, MLKL successfully co-precipitated with UBE2L3, whereas no such interaction was observed in the control group (Fig. 7). This interaction provides a molecular basis for UBE2L3-mediated MLKL ubiquitination and degradation. In conclusion, UBE2L3 directly interacts with MLKL, enhances its ubiquitination, and facilitates its degradation via the proteasome pathway. This mechanism likely plays a critical role in regulating MLKL stability and associated signaling pathways.

Discussion

Osteosarcoma (OS) is a highly malignant bone tumor with a complex pathogenesis involving multiple molecular and signaling pathway abnormalities [16, 17]. Current studies have identified several key factors and mechanisms contributing to OS initiation and progression, including gene mutations (e.g., TP53 and RB1) [18–21], gene amplifications (e.g., MDM2 and CDK4) [22, 23], abnormal epigenetic modifications [24], and dysregulated expression of non-coding RNAs [25]. Notably, these molecular alterations ultimately influence the cell death processes in OS, leading to aberrant tumor behavior [26, 27]. Ubiquitination, a critical post-translational protein modification, regulates various physiological processes, including the cell cycle, DNA repair, apoptosis, and tumor progression [28, 29]. Emerging evidence indicates that dysregulated ubiquitination is closely associated with OS cell proliferation, invasion, metastasis, and treatment resistance [30, 31]. Our previous studies have highlighted the pivotal role of necroptosis in OS progression, with the RIPK1/RIPK3/MLKL signaling pathway identified as its central regulatory axis. However, therapeutic strategies targeting RIPK1 and RIPK3 have yielded unsatisfactory outcomes. Additionally, the genetic knockout of RIPK family proteins has proven lethal in mice, underscoring the need for alternative therapeutic targets to modulate necroptosis [6, 12]. In this context, MLKL, as the downstream effector of necroptosis, has garnered increasing attention as a promising therapeutic target for OS treatment.

In addition, our preliminary research has confirmed that UBE2L3 expression is abnormally elevated in various osteosarcoma cell lines. Furthermore, transfection of UBE2L3 plasmid silencing can inhibit the proliferation, invasion, and migration abilities of U2OS and HOS osteosarcoma cells [32]. However, the expression of UBE2L3 in osteosarcoma and its role in the pathogenesis of osteosarcoma are still not well understood, and there are relatively few relevant literature and studies available. UBE2L3 is mainly involved in the ubiquitin-proteasome system (UPS) of cells, which is a major pathway responsible for protein degradation in cells [33]. This system plays a central role in maintaining cellular protein homeostasis, controlling cell cycle, immune response, and signal transduction processes [34]. Therefore, by modulating this system, UBE2L3 may play a key role in the occurrence and development of osteosarcoma. Previous studies have found that UBE2L3 can regulate the activation of the NF-κB signaling pathway. NF-κB is an important transcription factor that regulates the expression of multiple genes, including those involved in cell proliferation, apoptosis, immune response, and inflammation, all of which are closely related to tumor development [35]. Therefore, UBE2L3 may affect the biological behavior of osteosarcoma to some extent by regulating the NF-κB signaling pathway. In this study, we also found that UBE2L3 can ubiquitinate the MLKL protein, thereby reducing its expression, while silencing UBE2L3 can inhibit the ubiquitination of MLKL protein and stabilize its expression.

Mixed lineage kinase domain-like protein (MLKL) is an important effector molecule downstream of the necrosis-associated speck-like protein containing a CARD (NACHT, LRR and PYD domains-containing protein 3, NLRP3) pathway. It interacts with receptor-interacting serine/threonine-protein kinase 3 (RIPK3) and is phosphorylated by it, leading to conformational changes and exposure of the N-terminal oligomerization domain. Upon oligomerization, MLKL can insert into the cell membrane, increase membrane permeability, and ultimately cause cell lysis and necrosis [12]. Currently, the understanding of the mechanism of MLKL in the occurrence and development of osteosarcoma is limited. One study showed that compared to normal bone tissue, the mRNA and protein levels of MLKL were significantly downregulated in osteosarcoma tissues and cell lines. The low expression of MLKL may inhibit the activation of the cell death pathway and promote the unlimited proliferation of tumor cells [36]. Another study found that the fluoropyrimidine anti-tumor drug 5-fluorouracil can upregulate the expression of MLKL in osteosarcoma cells and induce phosphorylation-mediated aggregation of MLKL, thereby activating the downstream cell death process [37]. Overall, as a crucial molecule in regulating cell survival and death, the specific mechanism of MLKL in the occurrence and development of osteosarcoma needs further clarification. Continuing research on the relationship between MLKL and osteosarcoma will not only contribute to a better understanding of the pathogenesis of osteosarcoma and provide a theoretical basis for identifying new biomarkers and targeted therapies but also offer new insights for the development of drug treatments for osteosarcoma.

Furthermore, this study also discovered that interfering with UBE2L3 expression can inhibit the growth of osteosarcoma tumors in vivo. UBE2L3 is identified as one of the key enzymes involved in the ubiquitination process. Its main function is to transfer ubiquitin molecules to substrate proteins through an isopeptide bond under the action of ubiquitin ligases, thereby promoting the degradation of substrate proteins. Research indicates that UBE2L3 participates in the regulation of various biological processes, including cell growth, apoptosis, RNA transcription, DNA replication, and repair, by modulating the stability and activity of multiple genes [33]. Studies utilizing osteosarcoma xenograft models have found that UBE2L3 is highly expressed in osteosarcoma tissues compared to normal tissues. Furthermore, the expression

level of UBE2L3 is positively correlated with the rate of tumor growth and degree of necrosis. This suggests that UBE2L3 may be involved in the occurrence and development of osteosarcoma by modulating apoptosis in osteosarcoma cells [38]. Necroptosis is a specific form of cell death that occurs as an alternative mechanism when apoptosis pathway is impaired. Induction of necroptosis in tumor cells is considered as an effective anti-tumor therapeutic strategy. Research has found that during tumor necroptosis, UBE2L3 can regulate the formation of ripoptosome, a complex involved in necroptosis, by ubiquitinating specific ligands such as RIPK1 protein. This enables UBE2L3 to implement negative feedback regulation against excessive necroptosis and suppress exaggerated cell death in antiviral response and tumor immune surveillance [32]. This suggests that UBE2L3 may also be involved in the regulation of necroptosis in osteosarcoma cells. Therefore, UBE2L3 may play an important role in the occurrence and development of osteosarcoma by participating in the regulation of cell apoptosis and necroptosis. Elucidating the connection between UBE2L3 and osteosarcoma as well as cell death mechanisms will contribute to the development of targeted therapies against this molecule and provide new insights for precision treatment of osteosarcoma.

In summary, UBE2L3 can specifically bind to MLKL and accomplish downstream regulation of MLKL ubiquitination. This leads to a reduction in MLKL expression, thereby promoting necroptosis in osteosarcoma cells and inhibiting osteosarcoma growth.

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The original contributions presented in the study are included in the article/supplementary material, further inquiries can be directed to the corresponding authors.

Author contributions
XW Zhao and GQ Shan designed the study; DG Xing and HW collected, interpreted, and analyzed the data; ZG Xiong and WP Hui took part in drafting the manuscript or revising it; Mingzhi Gong gave final approval for the version to be published; and agreed to be accountable for all aspects of the work. All authors read and approved the final manuscript.

Funding
None.

Data availability
The original contributions presented in the study are included in the article/supplementary material, further inquiries can be directed to the corresponding authors.

Declarations

Ethics approval and consent to participate
Approval was obtained from the respective ethics committee (The Second Hospital of Shandong University) No.KYLL2024349. The procedures used in this study adhere to the tenets of the Declaration of Helsinki. The consents were given voluntarily by study participants and the approvals were obtained from all participants.

Consent for publication
Informed consent was obtained from all individuals participating in the study.

Competing interests
The authors declare no competing interests.

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