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Original Article

The deubiquitinase OTUB2 promotes cervical cancer growth through stabilizing FOXM1

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Abstract: Objectives: Ovarian tumor (OTU) domain-containing ubiquitin aldehyde-binding protein Otubain2 (OTUB2) is an important cysteine protease with deubiquitinase activity in the OTU family. However, the role of OTUB2 in cervical cancer (CC) has not been investigated. Methods: OTUB2 expression was analyzed employing the CC data from The Cancer Genome Atlas (TCGA) database. Western blot and qRT-PCR analysis were performed to identify OTUB2 expression in CC. The oncogenic function of OTUB2 was identified through a series of *in vitro* and *in vivo* experiments. Tandem Mass Tag™ Quantitative Proteomics examination was used to identify potential targets of OTUB2. Results: OTUB2 was overexpressed in CC and was related to poor prognosis of patients. In our in-house cohort, we also showed that OTUB2 was overexpressed in tumor tissues of CC compared to para-tumor. Knockdown of OTUB2 suppressed CC cell growth whereas OTUB2 upregulation fostered the proliferation of cancer cells. Forkhead box M1 (FOXM1) was found to be a target of OTUB2. FOXM1 can be positively regulated by OTUB2 in CC cells. In human CC tissues, protein level of FOXM1 was positively correlated with OTUB2. FOXM1 was found to play a critical role in OTUB2-mediated CC cell growth. Mechanistically, OTUB2 could bind FOXM1 and deubiquitinate FOXM1 to stabilize it. Conclusion: OTUB2 promotes CC progression through deubiquitinating and stabilizing FOXM1.

Keywords: OTUB2, FOXM1, cell proliferation, cervical cancer

Introduction

Cervical cancer (CC) is the fourth most common cancer in women, with an increasing incidence [1]. Patients with persistent, recurrent, or distant metastasis are not suitable for curative therapies and have a poor prognosis. For CC patients, surgery, chemotherapy, and radiation have been the main treatment modalities. To date, novel therapeutics have been applied that provided new treatment options for women with advanced and/or recurrent disease. Immunotherapy has become an effective therapeutic strategy to treat CC with the approval of pembrolizumab for the second-line treatment of patients with advanced or recurrent disease [2]. It is still urgent to develop novel therapeutic strategies to improve the prognosis of CC patients, which requires understanding of the molecular mechanisms of its development and progression.

Ubiquitination of proteins can be reversed by deubiquitylating enzymes (DUBs, also known as deubiquitinases), which cleave the ubiquitin modifications from substrates. Ovarian tumor (OTU) domain-containing ubiquitin aldehyde-binding protein Otubain2 (OTUB2) is a cysteine protease with deubiquitinase activity in the OTU family [3]. Multiple studies have demonstrated that OTUB2 plays essential roles in multiple cancer types including colorectal cancer (CRC) [4], non-small cell lung cancer (NSCLC) [5], gastric cancer (GC) [6], and intrahepatic cholangiocarcinoma (ICC) [7]. Recently, a study also suggested that OTUB2 enhances metastasis through Hippo-independent upregulation of YAP/TAZ signaling. Furthermore, OTUB2 was found to interact with and deubiquitinate YAP/TAZ [8]. But the function and the underlying mechanism of OTUB2 in CC remain elusive.

Here, we found the role and the clinical relevance of OTUB2 in CC. Our data for the first time

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unraveled the oncogenic function of OTUB2 in CC and demonstrated that OTUB2 promotes CC growth through stabilizing FOXM1, a proliferation-related transcription factor of the forkhead box proteins superfamily. Hence, our study provides novel insight into the therapeutic application of targeting OTUB2 in CC treatment.

Materials and methods

Human samples collection

Cervical cancer samples were obtained from the Jiangxi Maternal & Child Health Hospital. 53 patients who underwent surgical resection were confirmed to have cervical cancer and written informed consent were obtained. The research has been approved by the Ethical Committee of the Jiangxi Maternal & Child Health Hospital. Collected tumor and peritumor tissues were analyzed using qRT-PCR and western blot.

Cell culture and treatment

HeLa, SW756, SiHa, CaSKi, C33A and primary normal human keratinocytes (NHKs) cells were obtained from the Cell Bank of Type Culture Collection of Chinese Academy of Sciences and the Shanghai Institute of Cell Biology in China. All cells were cultured in Dulbecco's Modified Eagle's Medium (DMEM, Gibco) supplemented with 10% fetal bovine serum (FBS, HyClone, USA) in a humidified incubator containing 5% CO_2 at 37°C. Short tandem repeat analysis of all cell lines authenticity has been confirmed.

Western blot

Western blot was performed as previously described. RIPA buffer (Beyotime, China) adding protease inhibitor (Beyotime, China) was used to lyse cells and obtain total protein. SDS-PAGE and polyvinylidene fluoride membranes were employed to separate protein samples and transferred, respectively. Then, 5% nonfat milk was used to block membranes, followed by primary antibody incubation. Secondary antibody (Proteintech, China) was used for incubation for 1 hour at 37°C. Protein bands intensity was detected using Quantity One software (Bio-Rad, USA). The following antibodies were used: anti-OTUB2 antibody (1:1000; PA5-99680, Invitrogen), anti-FOXM1 antibody (1:1000, ab207298, Abcam), anti-ubiquitin antibody (1:1000, ab140601, Abcam).

Quantitative real-time PCR (qRT-PCR)

The qRT-PCR was performed as described previously [9]. The standard Trizol reagent (Invitrogen, USA) was employed to extract total RNA of cells and tissue and PCR was performed employing SYBR Premix Ex Taq (TaKaRa Bio, Japan). Primer pairs were provided as follows: GAPDH, forward 5'-CCATGGGGAAGGTGAAGGTC-3' and reverse 5'-TGAAGGGGTCATTGATGGCA-3'; OTUB2, forward 5'-TTCTTCGGGACCATCCTGAAA-3' and reverse 5'-CCAGGTAGGAATAGCCCAAGG-3'; FOXM1, forward 5'-CGTCGGCCACTGATTCTCAAA-3' and reverse 5'-GGCAGGGGATCTCTTAGGTTC-3'.

Stable cell line construction

OTUB2 stably overexpressing CC cell lines were established using OTUB2 overexpressing lentivirus vector plasmid pCDF1 (Geenchem, China). The lentivirus with short hairpin RNAs targeting the OTUB2 was employed to set up CC cell lines with stable OTUB2 knockdown. Puromycin (Invitrogen) was added for three weeks to select lentivirus infected cells.

Cell counting kit-8 (CCK8) assay

10^4 CC cells were seeded per well in 96-well plates. After 24 h culturation at 37°C in 5% CO_2, the cells were separated into indicated groups with different treatments; 3 repetitions were performed in each group. 10 μl of CCK-8 solution (#C0037, Beyotime, China) was added to each well according to the manufacturer's instructions. After incubation for 1 h, the absorbance at wavelength of 450 nm was evaluated by a microplate reader.

5-ethynyl-20-deoxyuridine (EdU) assay

Cells were treated using EdU (Ribobio) for 2 h and performed according to the manufacturer's instruction. Then, 1× Apollo reaction cocktail was used to treat cells for 0.5 h. Hoechst 33342 (5 mg/mL) was used to stain the DNA contents of the cells for 20 minutes. The experimental results were imaged under a fluorescence microscope.

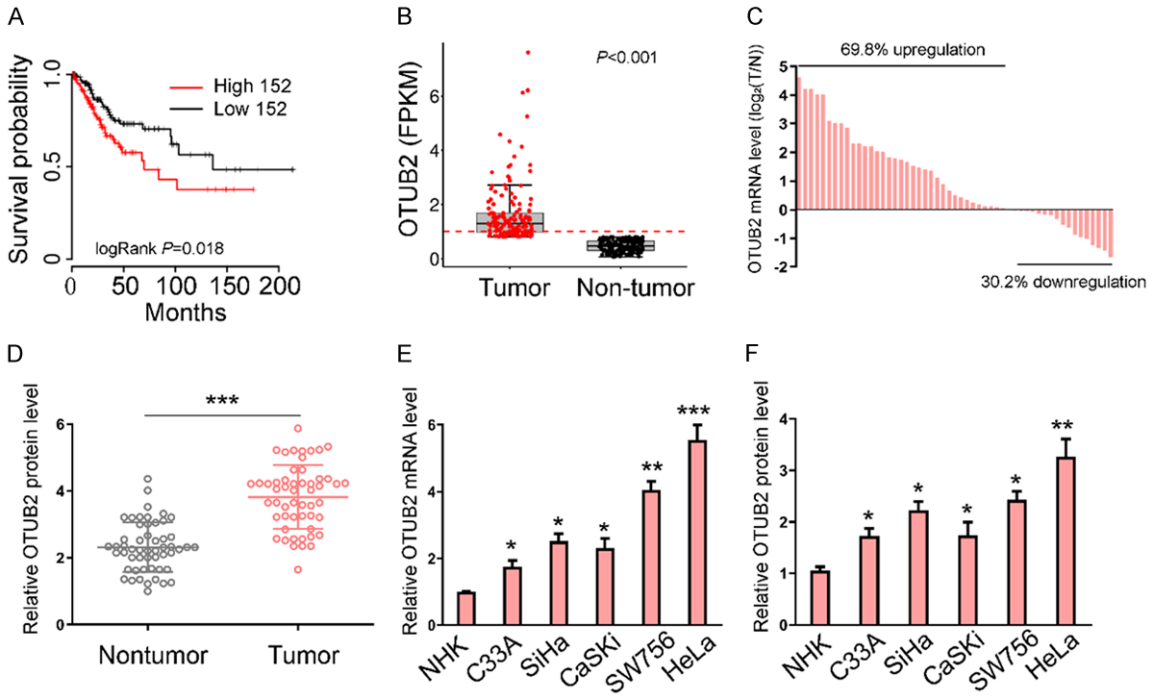

Figure 1. OTUB2 expression is elevated in human CC. A and B. TCGA database analysis of the prognosis and expression of OTUB2 expression in CC patients. C. qRT-PCR analysis of OTUB2 mRNA level in 53 paired CC tissues. D. Western blot analysis of OTUB2 protein level in 53 paired CC tissues. E and F. OTUB2 mRNA and protein expression analysis in CC cell lines and normal human keratinocytes (NHKs). Data are mean ± S.D. of three independent determinations. **P*<0.05, ***P*<0.01, ****P*<0.001 by t-tests.

Xenografts mouse model

The animal research in this study was approved by the Ethics Committee for Animal Experiments of the Jiangxi Maternal & Child Health Hospital. 4-week-old female BALB/c nude mice (Model Animal Research Center, China) were injected with CC cells (1×10^6) into the flanks. Tumor volumes were evaluated and recorded every 5 days. Tumor volume was determined using the modified formula, tumor volume = 1/2 length × width^2. At the 30th day, mice were sacrificed after 4 weeks and tumor weights were evaluated.

Statistical analysis

All data were analyzed by GraphPad Prism (version 8.0). The data are shown as the mean ± SD of three independent experiments. The differences between groups were analyzed using Student t test when two groups were compared or by one-way ANOVA when multiple groups were compared followed by Tukey's test. Overall survival of patients was analyzed by Kaplan-Meier plot and assessed using log-rank test. Pearson's correlation analysis was used to describe the correlation between variables. $P<0.05$ was considered significant.

Results

OTUB2 is highly upregulated in cervical cancer tissues and cell lines

To investigate the function of OTUB2 in CC, we first evaluated the expression of OTUB2 in CC using the data from the TCGA database and found that OTUB2 was highly expressed in CC as well as significantly related to a poor prognosis of CC patients (**Figure 1A** and **1B**). Next, we examined the mRNA and protein level of OTUB2 in 53 CC tissues from our in-house cohort employing qRT-PCR and western blot, respectively, and found that OTUB2 level was markedly elevated in CC tissues compared to the para-tumor tissues (**Figure 1C** and **1D**). Additionally, we assessed the mRNA and protein level in OTUB2 CC cell line and detected a notably increased in CC cell lines compared to primary normal human keratinocytes (NHKs) (**Figure 1E** and **1F**). Collectively, these results suggest that OTUB2 is aberrantly upregulated in CC.

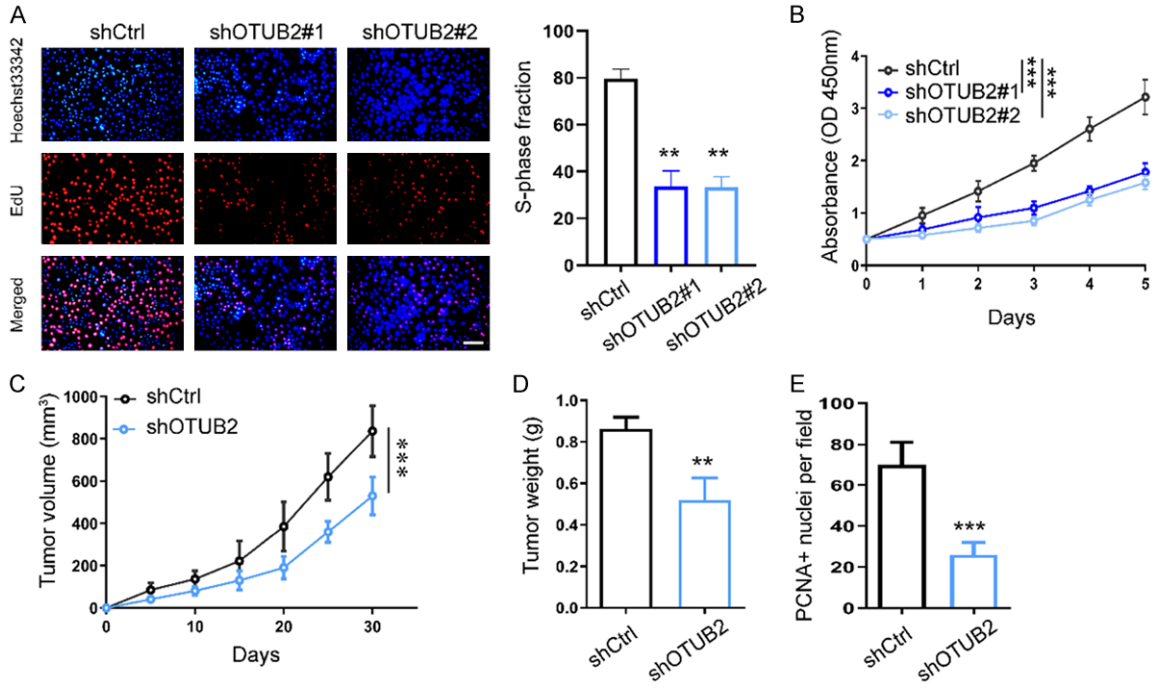

Figure 2. OTUB2 downregulation inhibits CC progression *in vitro* and *in vivo*. A and B. EdU and CCK-8 assay showing the proliferative ability of HeLa cells with OTUB2 knockdown compared with the control group. Scale bar, 100 μm. C and D. Tumor size and weight of nude mice in indicated group were measured. E. Quantification of immunohistochemical staining of PCNA in mouse tumor samples. Data are presented as mean ± S.D. of three independent determinations. **P*<0.05, ***P*<0.01, ****P*<0.001 by t-tests.

OTUB2 knockdown significantly inhibits CC growth in vitro and in vivo

To evaluate the role of OTUB2 in CC proliferation, we knocked down OTUB2 level by infecting HeLa cells with lentiviral plasmids containing shRNA sequence specifically targeting OTUB2. EdU assay and CCK-8 assay demonstrated a decreased cell proliferation with OTUB2 downregulation compared to the control group, suggesting that OTUB2 promoted cell growth (**Figure 2A** and **2B**). Furthermore, we established mouse xenograft tumor models to verify the effect of OTUB2 on CC growth in vivo. Consistent with our *in vitro* results, the downregulation of OTUB2 expression decreased the volume and weight of xenograft tumors (**Figure 2C** and **2D**). In addition, we confirmed that OTUB2 knockdown significantly suppressed the proliferation of tumor cells by performing immunohistochemical staining of proliferating cell nuclear antigen (PCNA) in tumor samples (**Figure 2E**). Collectively, these data suggest that OTUB2 downregulation can suppress CC growth.

OTUB2 overexpression fosters CC cell growth in vitro and in vivo

In addition to use the loss-of-function approach, we also verified the oncogenic function of OTUB2 by using gain-of-function approach. Specifically, we overexpressed OTUB2 in C33A cells by infecting cells with OTUB2-expressing plasmids. EdU assay and CCK-8 assay indicated that OTUB2 upregulation significantly promotes CC growth (**Figure 3A** and **3B**). Furthermore, we observed that OTUB2 upregulation increased the volume and weight of xenograft tumor (**Figure 3C** and **3D**). Moreover, we also confirmed that OTUB2 overexpression significantly promoted the proliferation of tumor cells as determined by PCNA staining of tumor samples (**Figure 3E**). Taken together, our results showed that OTUB2 upregulation enhances CC proliferation.

OTUB2 regulated FOXM1 protein level in CC cells

To understand the mechanism of OTUB2 function in CC progression, we performed tandem

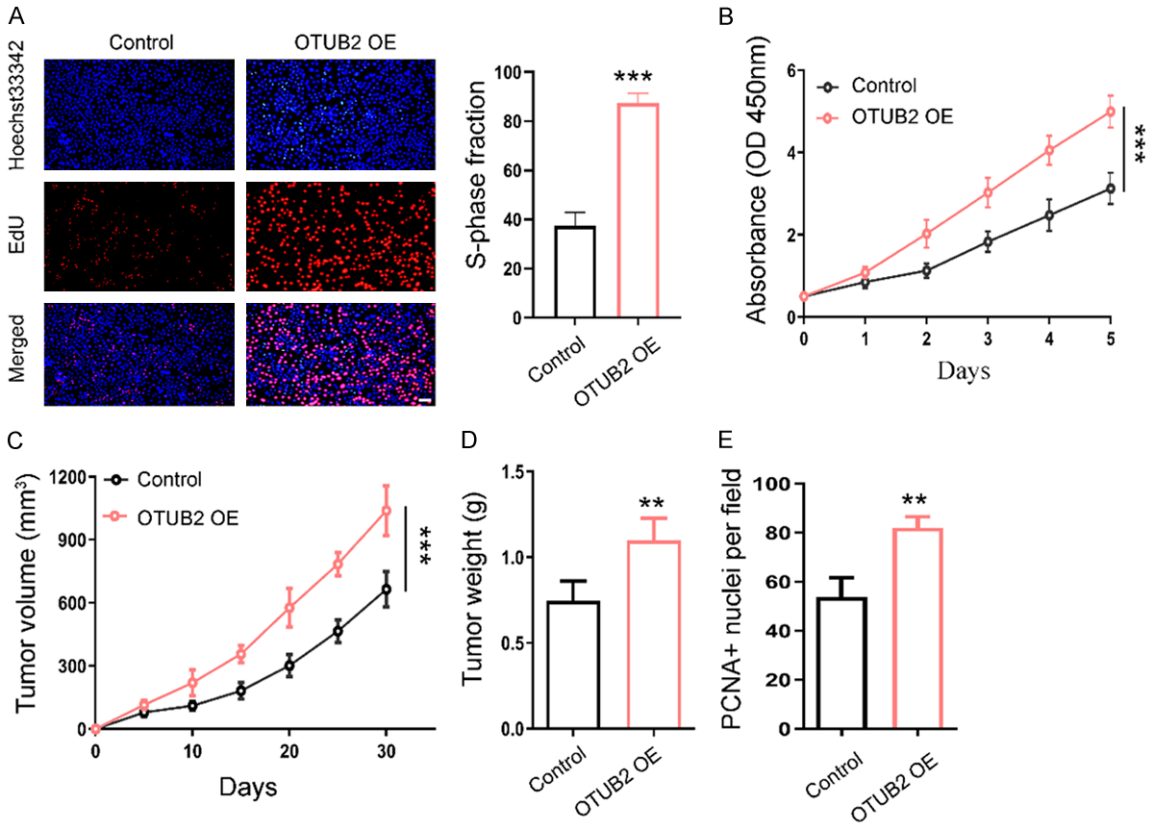

Figure 3. OTUB2 overexpression promotes CC proliferation *in vitro* and *in vivo*. A and B. EdU and CCK-8 assay evaluating the proliferative ability for CC cells with OTUB2 overexpression compared with the control group. Scale bar, 100 μm. C and D. Tumor size and weight of nude mice in indicated group were measured. E. Quantification of immunohistochemical staining of PCNA in mouse tumor samples. Data are presented as mean ± S.D. of three independent determinations. **P*<0.05, ***P*<0.01, ****P*<0.001 by t-tests.

mass tag (TMT)-mass spectrometry proteomics analysis to explore the protein expression profiles regulated by OTUB2. In OTUB2 knockdown HeLa cells, we identified 619 differentially expressed proteins (DEPs) compared to the control group, and FOXM1 was the most significantly downregulated protein (**Figure 4A**). FOXM1 is a transcription factor of the Forkhead family essential for cell proliferation and is overexpressed in multiple cancer types [10]. Hence, we performed western blot and found the protein level of FOXM1 was significantly upregulated in OTUB2-overexpressing C33A and SiHa cells. Consistent with this finding, FOXM1 protein level was markedly downregulated in OTUB2 knockdown HeLa and SW756 cells (**Figure 4B** and **4C**). Importantly, the mRNA level of FOXM1 was not regulated by OTUB2 (Supplementary Figure 1A-D). In addition, we also examined the protein level of OTUB2 and FOXM1 in 53 CC specimens and revealed a significant correlation between OTUB2 and FOXM1 protein levels (**Figure 4D**). In contrast, there was no correlation between OTUB2 and FOXM1 mRNA levels (**Figure 4E**). Together, these results suggested that OTUB2 regulates the expression of FOXM1 at protein level in CC.

FOXM1 is critical for OTUB2-promoted CC growth

To elucidate the potential role of FOXM1 in OTUB2-induced CC growth, we manipulated the expression levels of OTUB2 and FOXM1 in HeLa cells by transfecting OTUB2 shRNA-expressing and FOXM1-expression plasmids simultaneously and then measured the proliferation of these cells. Western blot analysis showed that FOXM1 overexpression reversed

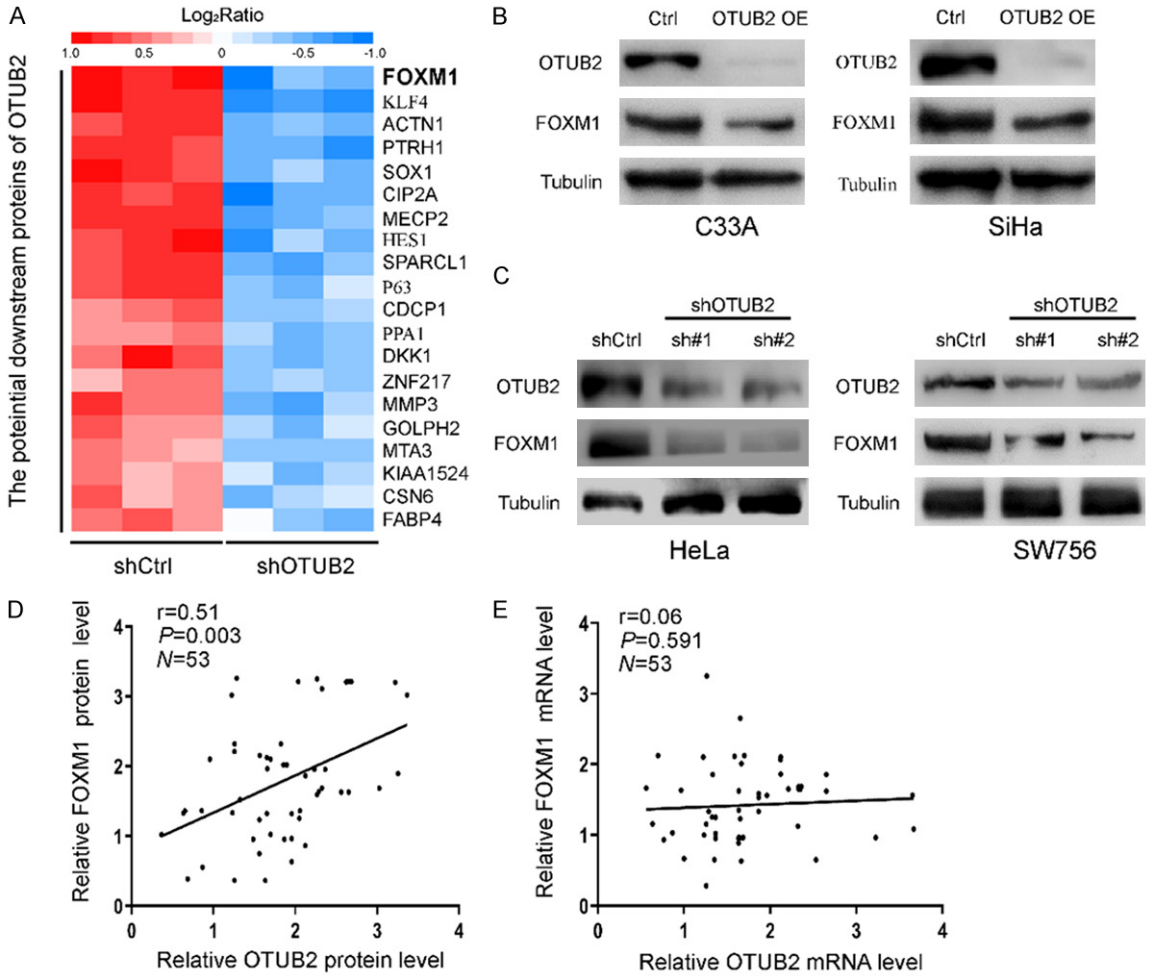

Figure 4. FOXM1 is significantly regulated by OTUB2 in CC. A. Heatmap of the TMT-mass spectrometry proteomics analysis in OTUB2 knockdown HeLa cells compared to the control group. B. Western blot evaluating the protein level of OTUB2 and FOXM1 in CC cells with OTUB2 overexpression compared to the negative control. C. Western blot detecting OTUB2 and FOXM1 protein level in CC cells with OTUB2 knockdown in contrast to the control group. D. Scatter plots showed positive correlation between OTUB2 and FOXM1 at the protein level in 53 CC tissues. E. Scatter plots indicated no significant correlation between OTUB2 and FOXM1 at the mRNA level in 53 CC tissues. Data are presented as mean ± S.D. of three independent determinations. **P*<0.05, ***P*<0.01, ****P*<0.001 by t-tests.

the decreased protein level of FOXM1 caused by OTUB2 knockdown (**Figure 5A**). Furthermore, OTUB2 knockdown inhibited cell growth, whereas FOXM1 upregulation effectively reversed the decreased cell proliferation, as determined by CCK-8 and EdU assays (**Figure 5B-D**). We further determined the physiologic function of OTUB2 and FOXM1 *in vivo* by generating mouse xenograft tumor models via injecting HeLa cells with OTUB2 knockdown and/or FOXM1 upregulation. Consistently, we found that OTUB2 knockdown inhibited tumor growth, which was reversed by FOXM1 upregulation (**Figure 5E** and **5F**), suggesting that FOXM1 regulates the growth-promoting function of OTUB2.

OTUB2 stabilizes FOXM1 through deubiquitylation

Since the data above suggested that OUTB2 regulates FOXM1 only at a protein level and OTUB2, a deubiquitinating enzyme, we hypothesized that OTUB2 might stabilize FOXM1 through deubiquitylation. To test this hypothesis, we first evaluated whether OTUB2 could interact with FOXM1 directly by performing confocal immunofluorescence assay and co-IP experiments. Our results showed that OTUB2 and FOXM1 co-localized in HeLa cells and OTUB2 co-immunoprecipitated with FOXM1 (**Figure 6A** and **6B**). These results are in line

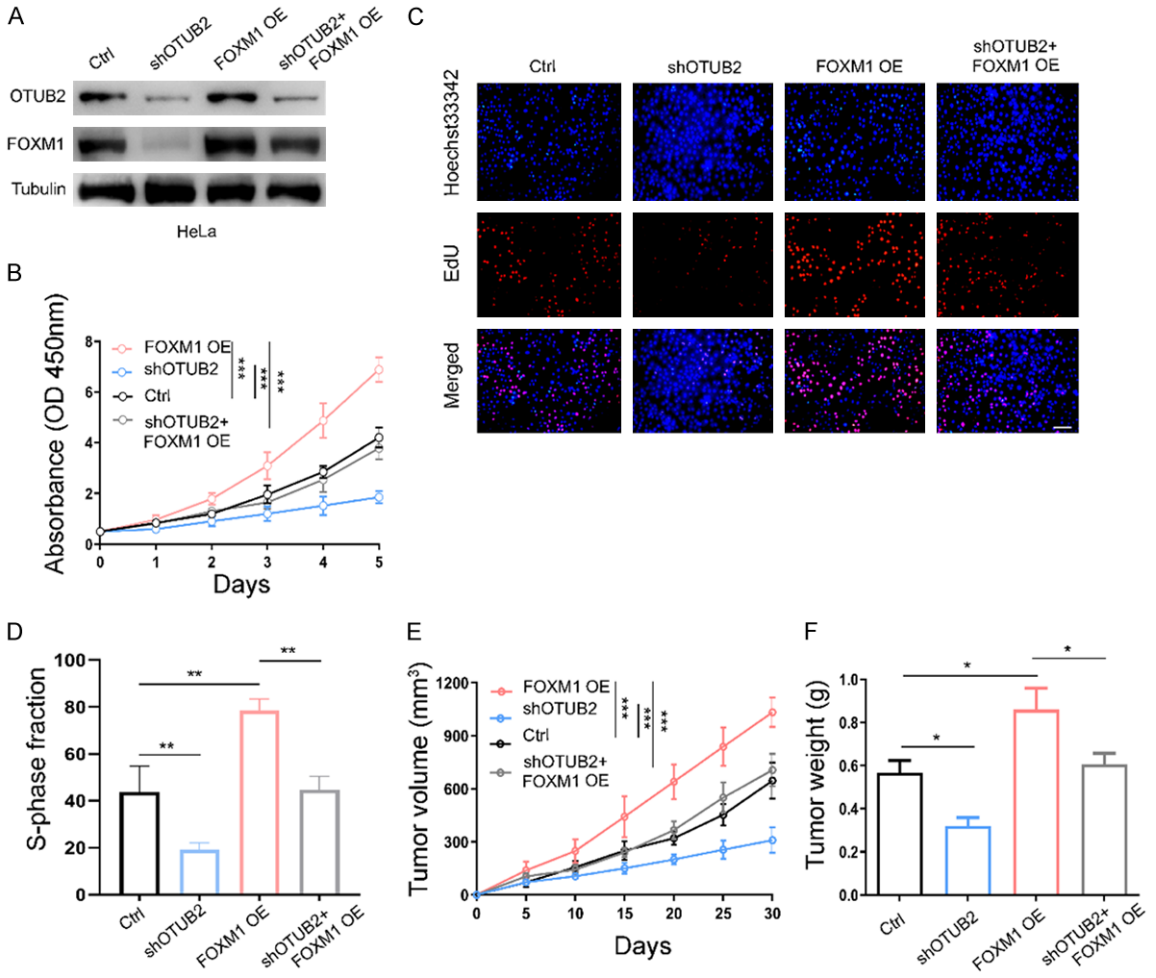

Figure 5. FOXM1 is critical for OTUB2-mediated CC growth. A. Western blot indicating that FOXM1 overexpression reversed the decreased FOXM1 protein level in OTUB2 knockdown HeLa cells. B-D. CCK8 and EdU assay indicating that OTUB2 knockdown inhibited cell growth whereas FOXM1 overexpression reversed this trend. Scale bar, 100 μm. E and F. Tumor sizes and weights of nude mice (n = 20, 5 mice per group) were recorded in indicated groups. Data are presented as mean ± S.D. of three independent determinations. **P*<0.05, ***P*<0.01, ****P*<0.001 by t-tests.

with recent reports that several DUBs can interact with FOXM1 and inhibit the ubiquitin-mediated proteasomal degradation of FOXM1 [11, 12]. Furthermore, we used proteosome inhibitor MG132 and translation inhibitor cycloheximide (CHX) to determine whether OTUB2 is involved in the process of FOXM1 ubiquitin-mediated proteasomal degradation and found that OTUB2 overexpression markedly increased the half-life of FOXM1 in HeLa cells (**Figure 6C**). Consistently, OTUB2 knockdown had no effect on FOXM1 protein level in the presence of MG132 compared with the control group (**Figure 6D**). Moreover, we performed deubiquitylation experiments to directly show that OTUB2 knockdown significantly increased the ubiquitination level of FOXM1 whereas OTUB2 upregulation led to the oppositive effect (**Figure 6E**). Therefore, we conclude that OTUB2 can deubiquitinate FOXM1 and prevent FOXM1 degradation by the ubiquitin-mediated proteasomal pathway.

Discussion

In this study, we for the first time demonstrated the important role as well as the underlying molecular mechanism of OTUB2 in regulating CC progression. We found that OTUB2 was significantly upregulated in CC tissues and could promote CC growth *in vitro* and *in vivo*. Importantly, we identified the involvement of

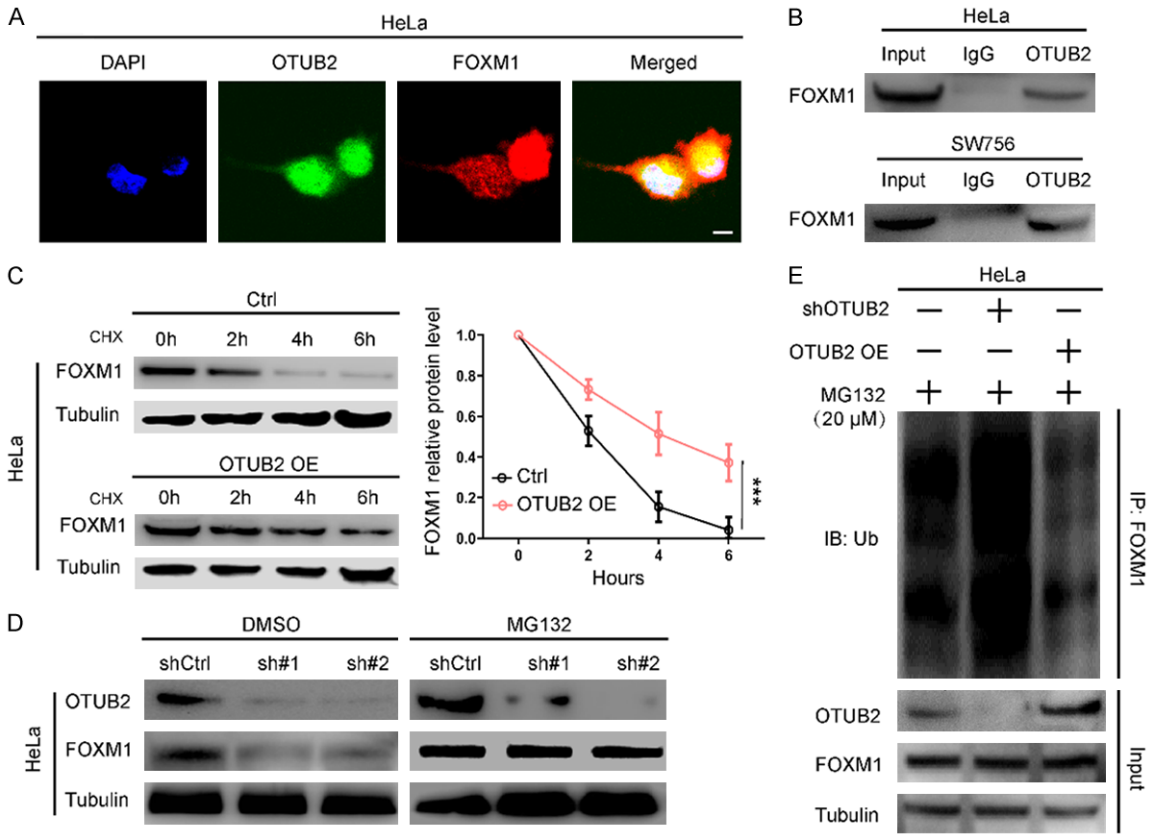

Figure 6. OTUB2 inhibits FOXM1 ubiquitin-proteasomal pathway through deubiquitylation. A. Confocal immunofluorescence assay was used to detect the protein expression of OTUB2 and FOXM1. Scale bar, 5 μm. B. co-IP experiments showing the interaction between endogenous OTUB2 and FOXM1 in CC cells. C. HeLa cells were treated with 30 μM cycloheximide (CHX) and western blot was used to detect FOXM1 expression in indicated time points. D. 20 μM MG132 was used to treat HeLa cells and FOXM1 level was evaluated using western blot. E. In HeLa cells, OTUB2 overexpression or knockdown impacts the ubiquitination level of FOXM1 protein. Data are presented as mean ± S.D. of three independent determinations. **P*<0.05, ***P*<0.01, ****P*<0.001 by t-tests.

FOXM1 in OTUB2-promoted CC growth and demonstrated that FOXM1 was indispensable for the function of OTUB2. Mechanistically, we revealed that OTUB2 acts as a deubiquitinase and interacts with FOXM1 to deubiquitylate FOXM1 to prevent its degradation through the ubiquitin-mediated proteasomal pathway. Our findings suggest the therapeutic potential of targeting OTUB2 for the treatment of patients with CC.

Ubiquitination is one of the most important posttranslational modifications of proteins, and it modulates a variety of cellular processes. Increasing evidence has confirmed the important role of deubiquitinating enzymes in cancers. Here, we focused on OTUB2, a member of the OTU subfamily of deubiquitinase. Previous studies have shown that OTUB2 is correlated with poor prognosis in multiple cancers [3] but the role of OTUB2 in CC remained elusive. Using the TCGA database and our in-house CC samples, we found OTUB2 is also correlated with the poor prognosis of CC patients and is significantly overexpressed in CC tissues. Though a series of gain- and loss-of-function assays, we verified the growth-promoting role of OTUB2, which is consistent with previous studies [5, 13].

OTUB2 was demonstrated to be a key regulator in multiple malignancies [5, 13]. A recent study showed that OTUB2 drives homologous recombination repair in endometrial cancer via YAP/TAZ-mediated Rad51 expression [14]. Another study also suggested that OTUB2 modulated the protein expression of YAP1/TAZ to enhance cell growth [5]. However, the downstream tar-

get of OTUB2 in CC has not been reported. In this study, we found OTUB2 regulates the expression of FOXM1. FOXM1 is a critical proliferation-associated transcription factor that is widely expressed during the cell cycle [15, 16]. FOXM1 is involved in the processes of cell proliferation, tumorigenesis, and self-renewal [17, 18]. In multiple cancers, FOXM1 is upregulated and is associated with a poor prognosis of patients [19, 20]. In our study, we found a positive correlation between OTUB2 and FOXM1 protein levels in CC and that FOXM1 was essential for OUTB2-enhahced CC progression. However, there was no correlation between OTUB2 and FOXM1 at mRNA level in CC. In support of this finding, a previous study has reported that FOXM1 can be stabilized by DUB [12]. Thus, we speculated that FOXM1 could be deubiquitinated by OTUB2.

As expected, our data displayed that OTUB2 interacted with FOXM1 in CC cells. Additionally, we also found that OTUB2 overexpression effectively prolonged that half-life of FOXM1. Consistently, MG132 treatment attenuated the effect of OTUB2 on FOXM1 protein level. Together, these data indicated that OTUB2 could affect the ubiquitin-mediated proteasomal degradation of FOXM1. Importantly, we directly confirmed that OTUB2 can inhibit the ubiquitination of FOXM1 to prevent the degradation of FOXM1, which is consistent with a recent report that OTUB2 regulates the glycolysis of CRC and acts as a negative regulator of PKM2 ubiquitination [4].

In conclusion, we present the first evidence that the OTUB2-FOXM1 axis is upregulated in CC and that OTUB2 promotes CC progression. OTUB2-FOXM1 axis may serve as a therapeutic target for CC treatment.

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Disclosure of conflict of interest

None.

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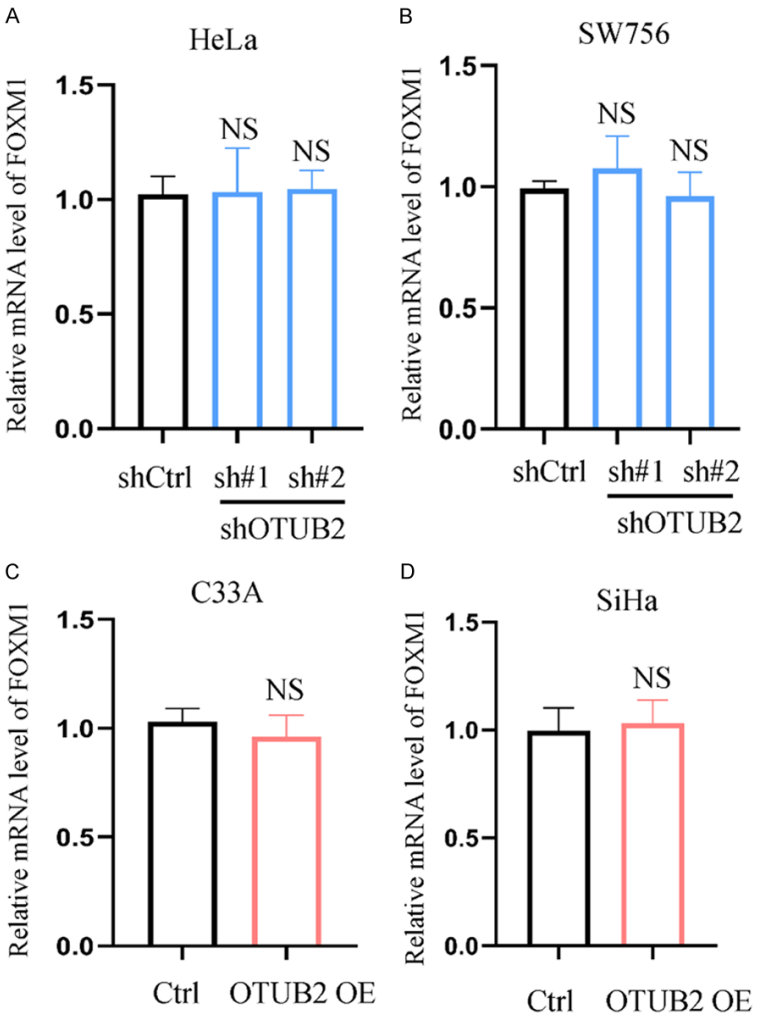

Supplementary Figure 1. A-D. qRT-PCR was performed. ***P*<0.01, ****P*<0.001 by t-tests, scale bar, 100 μm.