

Original Article

Ubiquitin-like protein FAT10 promotes osteosarcoma glycolysis and growth by upregulating PFKFB3 via stabilization of EGFR

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Abstract: Osteosarcoma is a major cause of cancer-related deaths in adolescents. While it thrives in a state of malnutrition, the mechanism of metabolic stress adaptation via metabolic reprogramming is unclear. Here, we found that the level of FAT10, a ubiquitin-like protein, was significantly higher in tumors than in adjacent normal tissues. Moreover, high FAT10 levels were closely related to increased malignancy and shorter survival time in osteosarcoma patients. Multivariate analysis also showed that FAT10 overexpression was an independent predictor of poor prognosis. Functional assays indicated that FAT10 promoted osteosarcoma cell proliferation by inducing glycolysis. In addition, FAT10 knockdown reduced the level of PFKFB3, a positive regulator of glycolysis in many cancers. A positive correlation was found between FAT10 and PFKFB3 levels in osteosarcoma tissues, further indicating that FAT10 induced an increase in glycolysis and that cell growth depended on PFKFB3. Interestingly, FAT10 regulated PFKFB3 expression by directly binding to EGFR and inhibiting its ubiquitination and degradation. These results shed light on the mechanisms responsible for osteosarcoma cell survival in the malnourished tumor microenvironment. Further, the results provide insights into the role of FAT10 in the adaptation of osteosarcoma cells to metabolic stress.

Keywords: Osteosarcoma, FAT10, EGFR, PFKFB3, glycolysis, growth

Introduction

Osteosarcoma (OS) often occurs in teenagers and is highly malignant [1]. Although the diagnosis and treatment of OS have progressed in recent years, the patient prognosis is still poor [2-4]. This is due to rapid OS cell proliferation, frequent early metastasis, and chemoresistance [5]. Therefore, to reduce the mortality of OS patients, strategies for early prevention are urgently required. At present, a major obstacle is our limited understanding of OS reprogramming in the unique tumor microenvironment [6]. Thus, further clarification of the molecular events underlying OS pathogenesis may assist in improving disease prognosis.

FAT10 is involved in multiple biological functions, including cell-mediated immunity, signal

transduction, protein translocation, and cell cycle regulation [7]. Previous studies have demonstrated that FAT10 plays a key role in tumor progression [8], and is highly expressed in hepatocellular carcinoma [9], colon cancer [10], pancreatic cancer [11], and gastric carcinoma [12]. FAT10 overexpression promotes colon cancer cell invasion and metastasis by activating the NF- κ B-CXCR4/7 signaling pathway [13]. The absence of FAT10 expression is associated with decreased NF- κ B activation by TNF- α , and inhibits tumor cell proliferation [14]. Although FAT10 expression is increased in OS, its pathogenic role has not yet been clarified.

The abnormal glycolysis pathway is a vital feature of tumor cells, and promotes tumor cell proliferation [15]. Even under normoxic conditions, tumor cells maintain an active glycolytic

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metabolism via a phenomenon called the Warburg effect [16]. The Warburg effect promotes glucose metabolism in tumor cells, which provides an energy source for tumor growth, enhances tumor cell tolerance to ischemia and hypoxia, prevents apoptosis via the inhibition of oxidative phosphorylation, and provides material for the metabolism of tumor cells during rapid growth [17, 18]. Increased glycolysis plays a key role in the rapid growth of OS. PFKFB3, a recently characterized glycolytic rate-limiting enzyme, is highly expressed in gastric cancer [19], colorectal cancer [20] and lung cancer [21] tissues. Silencing PFKFB3 expression significantly inhibits tumor cell growth [22]. Interestingly, we observed that FAT10 downregulation in OS cells decreases the level of glycolysis and inhibits PFKFB3 expression, but the underlying molecular events are unclear. However, we reasoned that the effect of FAT10 on OS cell glycolysis may be related to PFKFB3. Notably, it has been reported that EGFR activates PFKFB3 in tumor cells [23], thus regulating the level of glycolysis [24]. The function of FAT10 is protein polyubiquitination and targeting to proteasome degradation, but it is unclear whether FAT10 is involved in EGFR ubiquitination in OS.

In this study, we aimed to explore the role of FAT10 in the development of OS, and to evaluate its functional relationship with PFKFB3 and EGFR.

Materials and methods

Tissue samples

The OS tissues and their adjacent tissues were collected from the Second and First Affiliated Hospital of Nanchang University. The samples used for western blot and qRT-PCR assays were stored at -80°C , while the samples used for immunohistochemistry were stored in 4% paraformaldehyde tissue fixative at room temperature. The protocol was approved by the ethics committee of Nanchang University, and informed consent was obtained from all patients.

Cell lines and culture

The human OS cell lines Saos-2, MG-63, 143B, and U2-OS and the human osteoblasts hfoBI-19 were purchased from the Chinese Academy of Sciences (Shanghai, China). All OS cell lines

were cultured in Dulbecco's modified Eagle's medium (DMEM), while hfoBI-19 cells were cultured in DMEM/F12 medium. Then, 10% FBS and 1% penicillin and streptomycin were added to the above media. All cell lines were cultured at 37°C and 5% CO_2 in a 95% humidity cell incubator.

Immunohistochemical staining

OS and adjacent tissues were fixed, paraffin-embedded, sectioned, dewaxed, hydrated, and incubated with 3% H_2O_2 at room temperature for 5 min to eliminate endogenous peroxidase activity. Antigen repair was carried out, and then the sections were incubated at 4°C overnight with the appropriate antibodies (FAT10, PFKFB3) at a 1:200 dilution. Sections were then washed 3 times with PBS, and the corresponding secondary antibodies were added. After incubating for 2 h, the sections were washed 3 times with PBS, incubated with DAB basal solution (Dako, Denmark) for 30 min at room temperature, and then incubated with hematoxylin (Dako). Finally, dehydration, transparency, and sealing were carried out, followed by microscopic examination. The staining intensity and percentage of positive cells were scored semiquantitatively by two pathologists who were blind to the clinical parameters. The protein expression levels were subjectively graded as a function of relative nuclear staining intensity.

Quantitative real-time PCR (qRT-PCR)

Trizol reagent was used to extract total RNA from human OS tissues and cell lines. Then, the PrimeScript RT Reagent Kit was used for reverse transcription. Real-time fluorescent quantitative PCR amplification was then performed, as per the instructions of the SYBR Premix Ex Taq II Kit, on an Applied Biosystems 7500 real-time PCR system.

Western blot

OS cells and tissues were collected, and RIPA lysis buffer was used to extract the total protein. After measuring total protein concentration by BCA, protein buffer and proteins were boiled for 10 min. The same amount of protein (50 μg) was separated by 10% SDS-PAGE and transferred to PVDF membranes. After milk blocking, the membranes were incubated with

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antibodies against FAT10, PFKFB3, and EGFR (1:1000 dilution) overnight at 4°C, and then with the appropriate secondary antibodies. After washing the membranes with 1 × TBST, protein bands were detected by ECL, and their intensities were quantified using a chemiluminescence gel imaging analyzer.

shRNA plasmids and constructs

The plasmids for FAT10 and PFKFB3 overexpression, short hairpin RNA (shRNA), and the negative control were purchased from GenePharma (Shanghai, China). The overexpression plasmid, shRNA plasmid, and vector were used in accordance with the manufacturer's instructions. Transient transfection into OS cells was carried out for 48 h, and the effects of the transfections were verified. Then, the plasmids packed with lentivirus were stably transfected according to the detected multiplicity of infection (MOI), and the appropriate puromycin concentration (1-5 µg/ml, Sigma, USA) was applied for one week for clone selection.

Cell growth assays

EdU assays: After trypsin digestion and centrifugation, a single-cell suspension was prepared and counted. OS cells in complete culture medium were seeded in 96-well plates (1 × 10⁴ cells/200 µL) for 24 h. Cells were treated and cultured according to the EdU Kit instructions, and cell proliferation in each treatment group and the control group were examined by fluorescence microscopy.

CCK8 assays: OS cells (100 µL) in logarithmic growth phase (1 × 10⁴ cells) were seeded in 96-well plates. After 24 h of culture (37°C, 5% CO₂), the culture medium was changed and treatments were carried out. Then, cells were incubated with CCK8 for 1, 2, 3, 4, or 5 days. The absorbance at 450 nm was measured with a Bio-Rad instrument, and the average value of 5 wells per treatment was calculated.

Tumorigenicity assay

After approval by the Animal Care and Use Committee of Nanchang University, and in strict accordance with the Guidelines for Animal Care and Use of the National Institutes of Health, 6-week-old female BALB/C nude mice were used for xenotransplantation *in vivo*. Speci-

fically, 100 µL OS cells (5 × 10⁶) in logarithmic phase subjected to different treatments were subcutaneously injected into the back of nude mice through the thigh root. Tumor size was monitored over time, and tumor volume was measured every 5 days. After 35 days, mice were anesthetized, photographed, and examined. Tumor weight was measured and recorded.

Co-immunoprecipitation (Co-IP) and in vitro ubiquitination assay

Co-IP and *in vitro* ubiquitination assays were performed as previously described [25]. For Co-IP, cells were harvested and lysed on ice for 1 hour with addition of 500 µL IP buffer, the primary antibody (2 µL) was added and incubated for 2 h, the protein A/G plus-Agarose was added, after final washed, the pellet was resuspended in 20 µL of IP buffer. The samples were boiled 10 min, and aliquots were analyzed by SDS-PAGE. For *in vitro* ubiquitination assay, Osteosarcoma cells were transfected with shFAT10 and HA-FAT10 expression plasmids. The transfected cells were incubated for 30 hours and then exposed to MG132 (15 mmol/L) treatment for 4 hours before harvesting. The cell lysate was immunoprecipitated with UB and EGFR antibody.

Statistical analysis

All experiments were conducted at least three times, and the data were expressed as the mean ± SD. Data were analyzed by SPSS 26.0 and GraphPad Prism 7. Two-group comparisons were analyzed by unpaired Student's *t* test, and all data were compared by one-way analysis of variance (ANOVA). The correlation between the clinicopathological characteristics of OS patients and the expression level of FAT10 was analyzed by *chi* square test. Patient survival was analyzed by the Kaplan-Meier method, and *P* values <0.05 were considered statistically significant.

Results

FAT10 is upregulated in OS tissues and its expression is associated with poor prognosis in OS patients

Immunohistochemistry (IHC) showed that high FAT10 expression was observed in 64.29% (36/56) of OS tissues, but in only 16.07%

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(9/56) of non-cancer tissues (**Figure 1A, 1B**). qRT-PCR and immunoblotting confirmed that FAT10 was highly expressed in OS tissues (**Figure 1C, 1D**). In addition, our results confirmed that FAT10 expression was significantly higher in OS cell lines than in normal osteoblasts (**Figure 1E, 1F**). Furthermore, the relationship between FAT10 expression and pathological characteristics was analyzed. The level of FAT10 expression was closely related to the size of the tumor and the TNM stage, but had no obvious relationship with age, gender, degree of differentiation, or lymph node metastasis (**Table 1**). Finally, Kaplan-Meier survival curves confirmed the poor prognosis of patients with high FAT10 expression (**Figure 1G**).

FAT10 promotes OS cell proliferation in vivo and in vitro

To further explore the function of FAT10 in OS cells, we transfected shFAT10 and shNC plasmids into OS cells, and the transfection efficiency was verified by qRT-PCR and western blot (**Figures 2A, 2B, S1**). As shown in **Figure 2C-E**, FAT10 knockdown significantly reduced OS cell proliferation, as determined by CCK8, EdU, and clone formation assays. Next, the effect of FAT10 overexpression was explored (**Figure 3A, 3B**). CCK8, EdU, and clone formation assays showed that under these conditions, the proliferation ability of OS cells was increased (**Figure 3C-E**).

Next, we employed OS cells with FAT10 overexpression or knockdown in mouse tumor xenograft experiments. After 35 days, FAT10 knockdown in U2-OS cells (U2-OS/shFAT10) resulted in reduced tumor growth in nude mice, as compared to that in their respective controls (**Figure 2F, 2G**). Conversely, FAT10-overexpressing 143B cells (143B/HA-FAT10) yielded significantly larger tumors compared to control 143B cells (**Figure 3F, 3G**). Similarly, the average body weight of the U2-OS/shFAT10 group was significantly reduced (**Figure 2H**). On the contrary, the average body weight in the 143B/HA-FAT10 group increased significantly (**Figure 3H**) when compared to that of the respective controls. In conclusion, these data collectively indicated that FAT10 contributes to OS cell growth *in vitro* and *in vivo*.

FAT10 promotes glycolysis in OS cells

Aerobic glycolysis is a well-characterized metabolic shift that ubiquitously occurs in tumor

cells, including OS cells [26]. Therefore, we explored the role of FAT10 in OS cell glucose metabolism. As shown in **Figure 4A-C**, FAT10 knockdown dramatically decreased the cellular levels of glucose-6-phosphate (G6P), as well as glucose consumption and lactate production in OS cells. On the other hand, FAT10 overexpression led to the opposite effects (**Figure 4D, 4E**).

To further verify the effect of FAT10 on OS glycolysis, we tested the extracellular acidification rate (ECAR) of OS cells. As shown in **Figure 4F** and **4G**, FAT10 downregulation significantly reduced the glycolysis rate in OS cells, while FAT10-overexpressing OS cells exhibited increased ECAR. In conclusion, FAT10 promoted glycolysis in OS cells.

FAT10 positively regulates PFKFB3 protein levels

PFKFB3 is a new type of glycolytic kinase with high activity, and its abnormal expression has been reported in many tumors [22]. To verify whether it is also altered in OS, the level of PFKFB3 was analyzed in FAT10 knockdown cells. PFKFB3 expression was found to be significantly reduced under these conditions. Conversely, FAT10 overexpression was associated with increased PFKFB3 expression (**Figure 5A**). Furthermore, the mRNA expression of PFKFB3 was analyzed in 30 OS tissues. The results showed that PFKFB3 expression in OS cells was about twice that in the corresponding adjacent tissues (**Figure 5B**). Western blot analysis showed that of 30 OS specimens, 25 exhibited elevated PFKFB3 expression, while no significant differences to the controls were observed in the remaining 5 cases (**Figure 5C**). IHC assays confirmed increased FAT10 expression in OS tissues (**Figure 5D**). Moreover, scatter plots indicated that FAT10 and PFKFB3 expression levels were positively correlated in OS tissues (**Figure 5E** and **5F**). Together, these findings suggested that FAT10 promoted PFKFB3 expression, thus enhancing the rate of aerobic glycolysis in OS tissues.

PFKFB3 mediates FAT10-induced glycolysis and proliferation in OS cells

As discussed above, PFKFB3 was shown to act downstream of FAT10. Next, we sought to verify whether the effects of FAT10 on glycolysis and cell proliferation were mediated by PFKFB3. Initially, U2-OS cells with FAT10 knockdown

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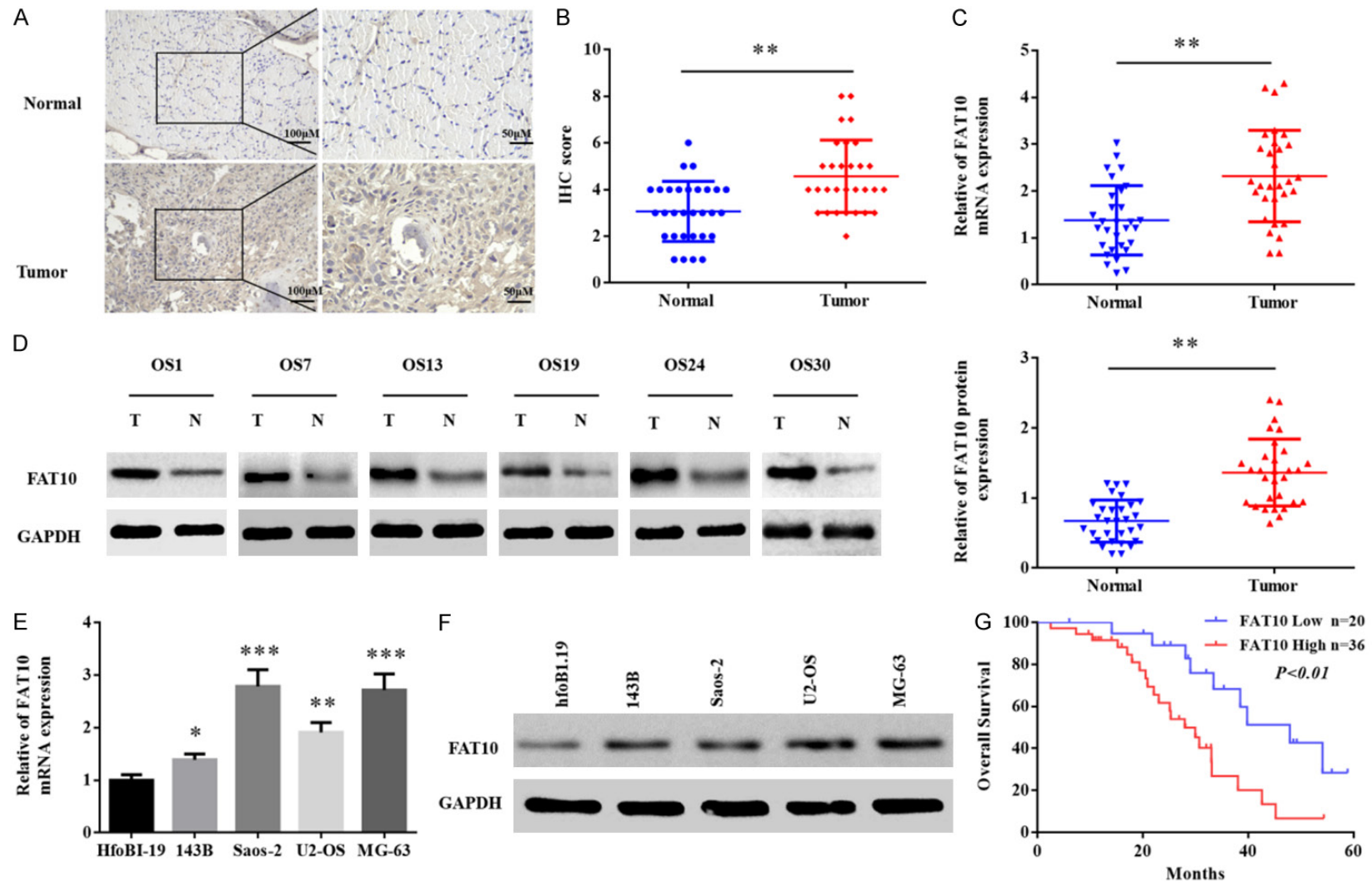


Figure 1. FAT10 was upregulated and associated with poor prognosis in osteosarcoma (OS) patients. A, B. Representative images and quantification of FAT10 immunohistochemical (IHC) staining in 56 paired OS and non-cancer tissues. High FAT10 expression was observed in 64.29% (36/56) of OS tissues, but in only 16.07% (9/56) of non-cancer tissues (** $P < 0.01$). C. qRT-PCR analysis showed that the level of FAT10 mRNA was significantly higher in OS tissues than in the corresponding adjacent tissue (** $P < 0.01$). D. Determination and quantification of FAT10 protein levels in OS tissues and paired non-tumor tissues by western blotting. GAPDH was used as a loading control (** $P < 0.01$, N = Normal, T = Tumor). E, F. qRT-PCR and western blot confirmed that OS cell lines exhibited a significantly higher FAT10 expression than normal hfoBl-19 osteoblasts (* $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$). G. Kaplan-Meier curves of overall survival in two groups of OS patients with low and high FAT10 expression, respectively.

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Table 1. Correlation between FAT10 expression and the clinicopathological characteristics of the osteosarcoma patients

Parameters	n	FAT10 expression		P value
		Low (n=20)	High (n=36)	
Age (years)				<i>P</i> =0.491
≤18	33	13	20	
>18	23	7	16	
Sex				<i>P</i> =0.873
Female	26	9	17	
Male	30	11	19	
Tumor size (cm)				<i>P</i> <0.001
<5	20	13	7	
≥5	36	7	29	
Location				<i>P</i> =0.449
Upper limb bone	27	11	16	
Lower limb bone	29	9	20	
Clinical stage				<i>P</i> <0.001
I/II	18	12	6	
III/IV	38	8	30	
Pathological differentiation				<i>P</i> =0.968
Well/Moderately	25	9	16	
Poor	31	11	20	
Lymph node metastasis				<i>P</i> =0.809
Absence	32	11	21	
Presence	24	9	15	
Distant metastasis				<i>P</i> =0.516
Absence	39	15	24	
Presence	17	5	12	
Recurrence				<i>P</i> =0.625
Absence	22	7	15	
Presence	34	13	21	

were transfected with HA-PFKFB3, and PFKFB3 overexpression was confirmed (**Figure 6A**). CCK8 assays demonstrated that U2-OS cell proliferation was partially restored by PFKFB3 overexpression (**Figure 6B**). G6P, glucose consumption, and lactate production were partially restored in U2-OS cells by the introduction of HA-PFKFB3 (**Figure 6C-E**).

Next, shPFKFB3 transfection was used to knock down PFKFB3 expression in FAT10-overexpressing OS cells (**Figure 6F**). The increase in cell proliferation induced by FAT10 overexpression was partially abolished by PFKFB3 silencing (**Figure 6G**). Consistently, the increase in G6P levels, glucose consumption, and lactate production induced by FAT10 overexpression in OS cells was partially abolished by PFKFB3 suppression (**Figure 6H-J**).

Taken together, these findings suggested that PFKFB3 mediated the FAT10-induced increase of aerobic glycolysis in OS cells.

FAT10 regulates PFKFB3 expression by activating EGFR in OS cells

Previous studies have shown that FAT10 interacts with other proteins. In order to further clarify the mechanism by which FAT10 affects PFKFB3 activity in OS, a direct binding between the two proteins was verified. Co-IP experiments showed that FAT10 and PFKFB3 did not directly interact (**Figure 7A**). It was reported that the transcription factor EGFR can regulate PFKFB3 expression [23], and other studies have confirmed that EGFR regulates the rate of glycolysis in different tumors [24]. Therefore, We hypothesized that FAT10 regulates PFKFB3 expression through EGFR. To test this hypothesis, we first verified whether FAT10 and EGFR could directly interact in OS cells. Interestingly, co-IP demonstrated an interaction between FAT10 and EGFR (**Figure 7B**). Moreover, to further demonstrate that FAT10 regulated PFKFB3 expression through EGFR, FAT10 silencing was found to decrease the protein level of EGFR in OS cells (**Figure 7C**). Conversely,

its expression increased after FAT10 overexpression, but its mRNA expression level remained unchanged (**Figure 7D**). Finally, we inhibited EGFR activity in FAT10-overexpressing OS cells. EGFR inhibition abolished FAT10-induced PFKFB3 upregulation (**Figure 7E**). Similarly, the CCK8 assay showed that EGFR inhibition reduced the FAT10-induced stimulation of cell proliferation (**Figure 7F**). Moreover, the increase in G6P levels, glucose consumption, and lactate production in FAT10-overexpressing U2-OS cells was partially abolished by cell treatment with the EGFR inhibitor (**Figure 7G-I**).

FAT10 stabilizes EGFR by regulating its ubiquitination in OS cells

The above experiments demonstrated that FAT10 could regulate PFKFB3 expression via

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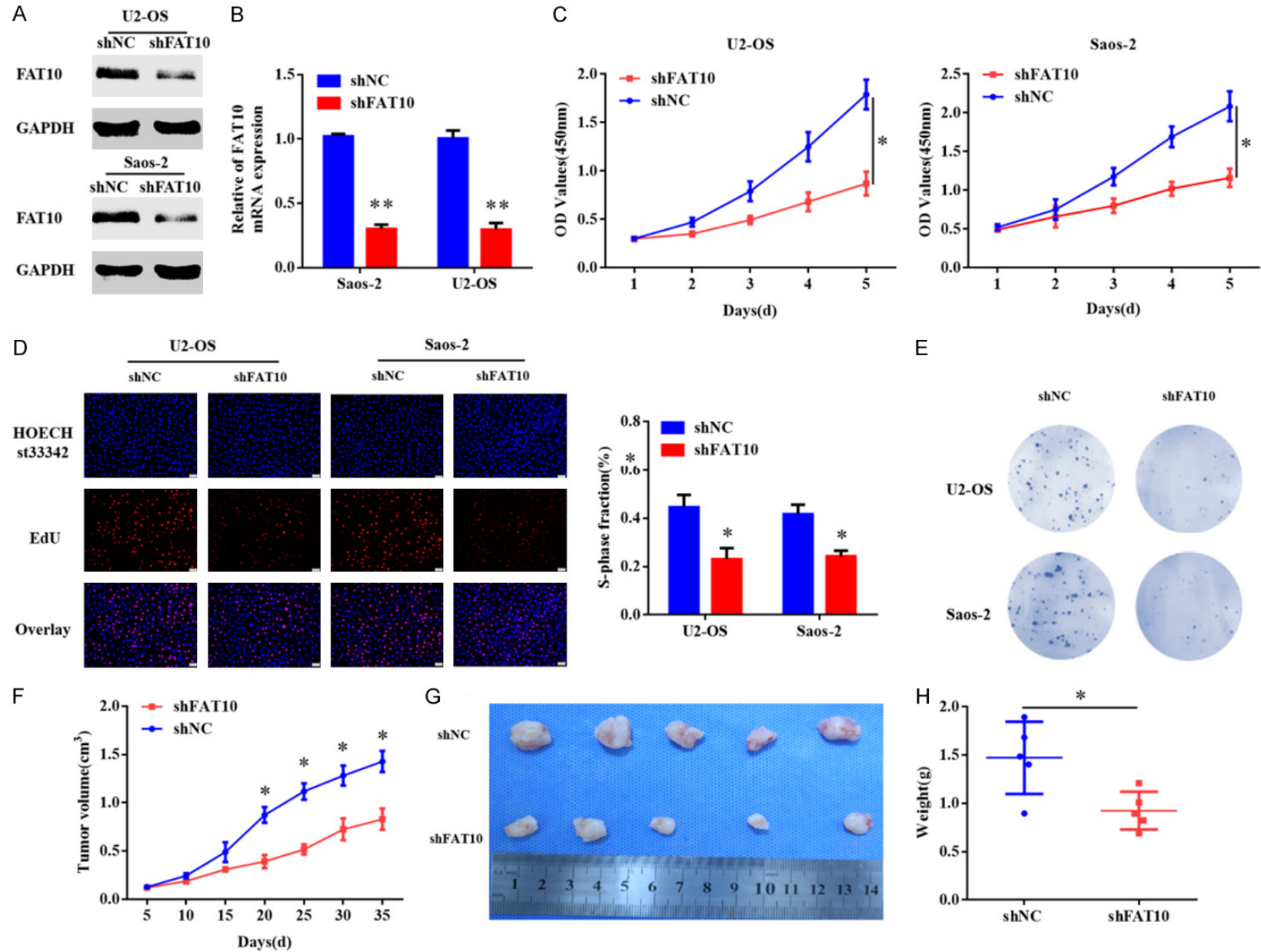
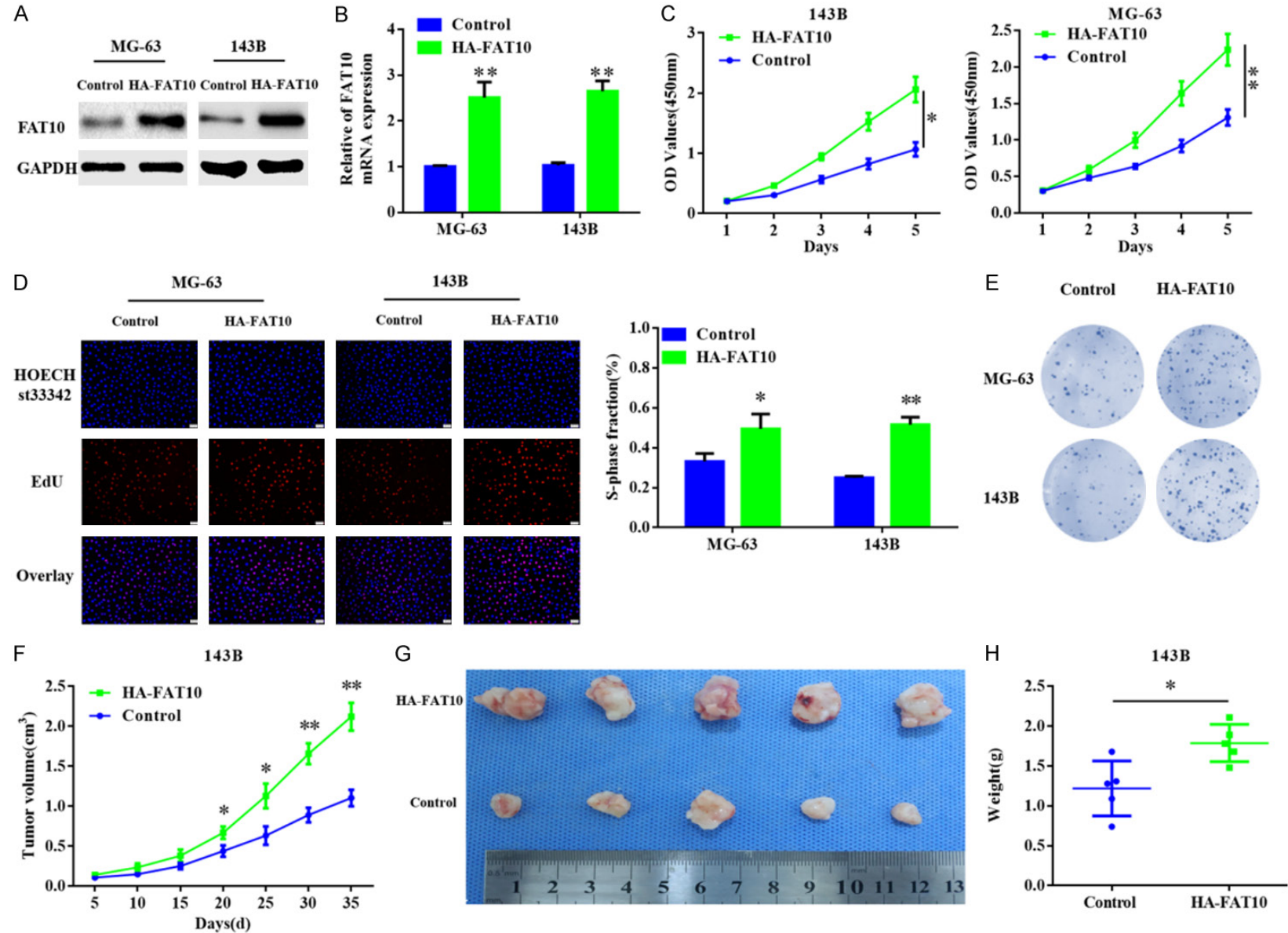


Figure 2. FAT10 knockdown inhibited osteosarcoma (OS) cell proliferation *in vivo* and *in vitro*. (A, B) Western blot and qRT-PCR analysis of FAT10 expression was reduced in U2-OS and Saos-2 cell lines that transfected with shNC and shFAT10 plasmids. (C-E) Impact of FAT10 knockdown determined by CCK8 (C), EdU (D), and clone formation (E) assays; OS cell proliferation was inhibited (*P<0.05). (F) U2-OS/shFAT10 and U2-OS/shNC cells were subcutaneously injected into nude mice, 2072 Am J Cancer Res 2020;10(7):2066-2082

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and tumor volumes were measured on the indicated days (* $P < 0.01$). (G, H) At the end of the experiment, tumors were dissected, photographed, and weighed ($n = 5$, * $P < 0.05$).



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Figure 3. FAT10 upregulation promoted osteosarcoma (OS) cell proliferation *in vivo* and *in vitro*. (A, B) HA-FAT10 plasmid was used to transflect MG-63 and 143B OS cell lines, and qRT-PCR and western blot confirmed FAT10 upregulation. (C-E) Effect of FAT10 upregulation in 143B and MG-63 cells, determined by CCK8 (C), EdU (D), and clone formation (E) assays; OS cell proliferation was significantly increased. (F) 143B/HA-FAT10 and 143B/Vector (control) cells were subcutaneously injected into nude mice, and tumor volumes were measured at the indicated times (** $P < 0.01$). (G, H) At the end of the experiment, tumors were dissected, photographed, and weighed ($n = 5$, * $P < 0.05$).

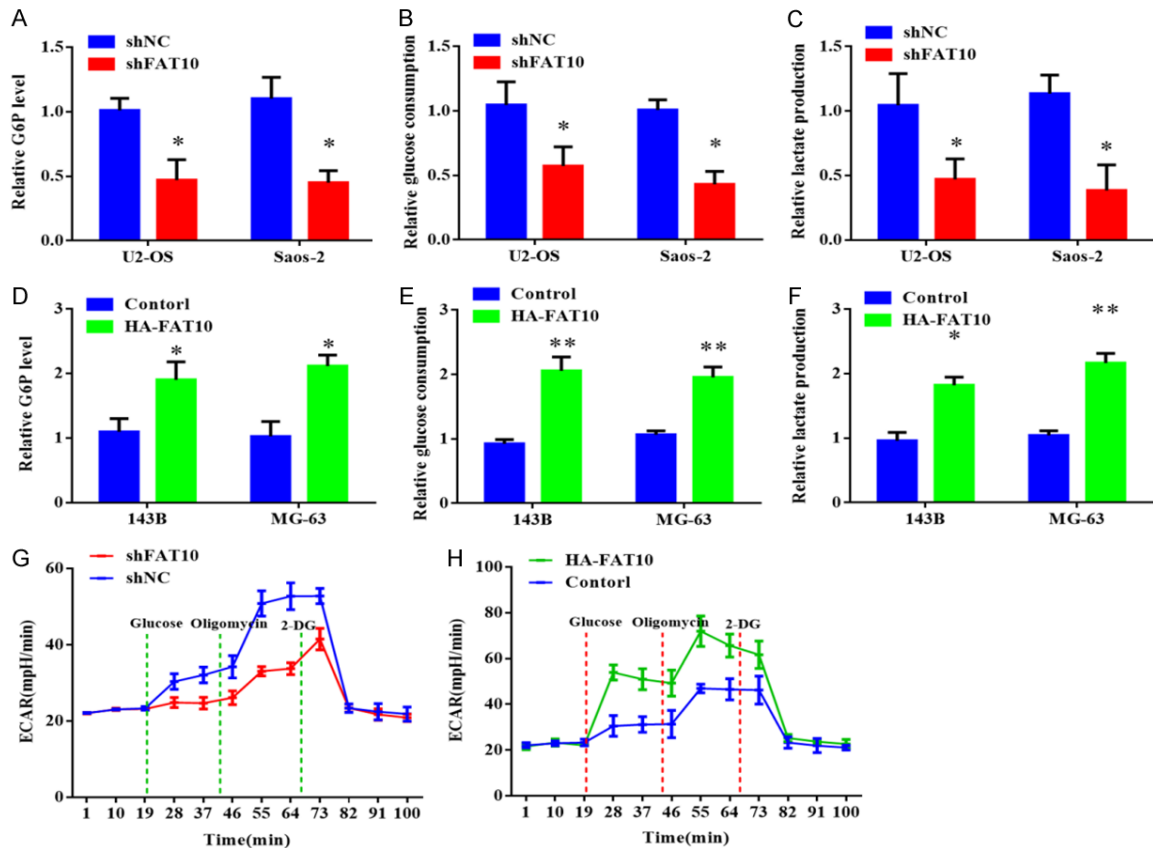


Figure 4. FAT10 promoted glycolysis in osteosarcoma (OS) cells. (A-C) Effect of FAT10 downregulation on the level of G6P (A), glucose consumption (B), and lactate production (C) in OS cells; all parameters were significantly decreased compared to controls. Each experiment was repeated 3 times (* $P < 0.05$). (D-F) Effect of FAT10 overexpression on the levels of G6P (D), glucose consumption (E), and lactate production (F) in 143B and MG-63 cells; all parameters were significantly increased compared to controls. (G, H) ECAR data showing the glycolytic rate in FAT10-silenced (G) or -overexpressing (H) OS cells. glucose (10 mM), the oxidative phosphorylation inhibitor oligomycin (1.0 μ M), and the glycolytic inhibitor 2-deoxyglucose (2-DG, 50 mM) were sequentially added to each well at the indicated time points. All measurements were normalized to the cell number based on crystal violet assay at the end of the experiment. * $P < 0.05$ vs. control.

direct binding to EGFR. Next, we explored the mechanism of EGFR regulation by FAT10. Previous studies showed that EGFR undergoes degradation via the ubiquitin-proteasome system (UPS) [27]. In addition, we previously demonstrated that FAT10 stabilizes the degradation of substrate proteins YAP1 [28] and β -catenin [9]. Consistently, treatment with the proteasome inhibitor MG132 led to substantial accumulation of endogenous EGFR protein in OS

cells (Figure 8A). Furthermore, co-localization experiments showed that endogenous EGFR directly bound to ubiquitin in pancreatic cancer cells (Figure 8B). These results demonstrated that EGFR is also degraded by the UPS in OS cells.

Next, to determine whether FAT10 was involved in the regulation of EGFR degradation, we transfected U2-OS and Saos-2 cells with the shFAT10

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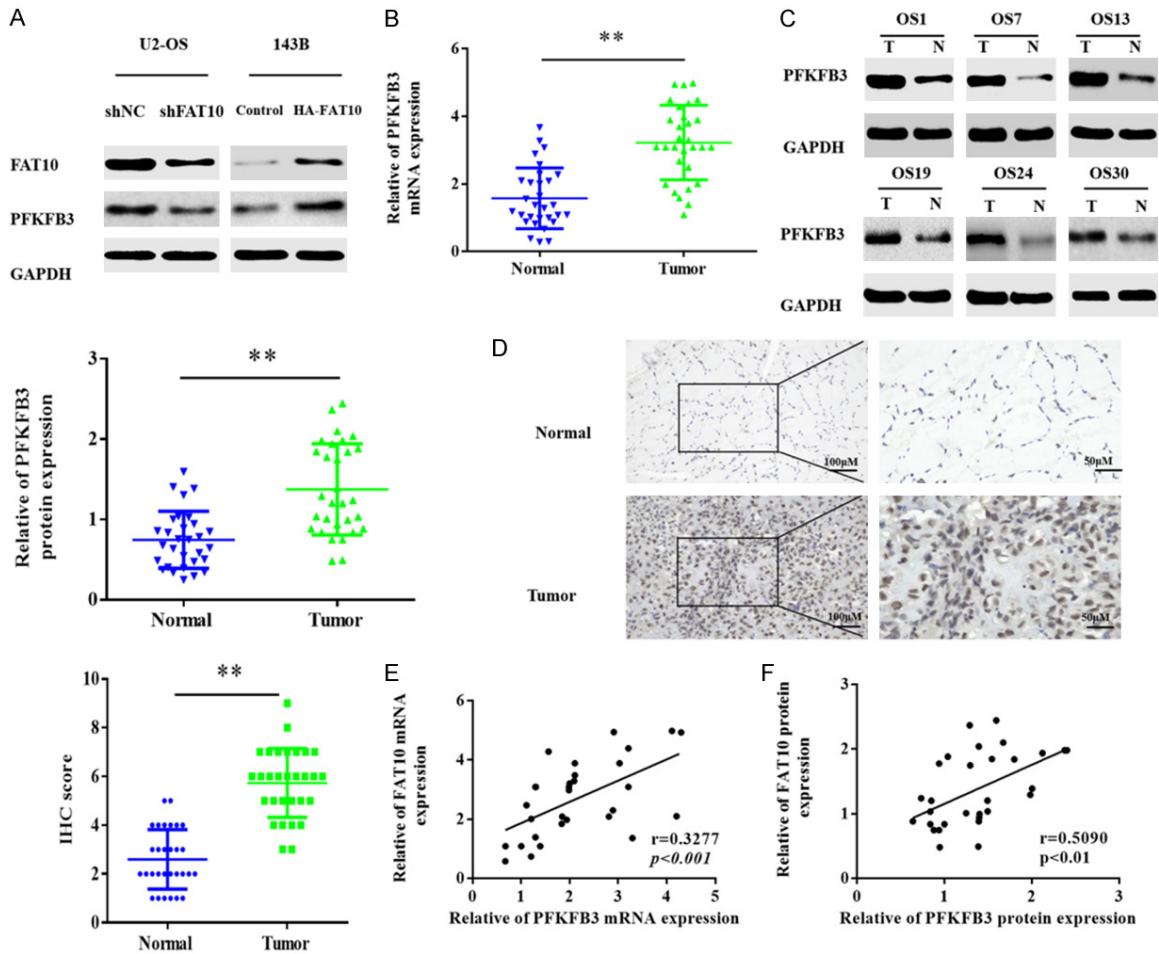


Figure 5. FAT10 positively regulated PFKFB3 protein levels. (A) FAT10 downregulation in osteosarcoma (OS) cells decreased PFKFB3 expression, as determined by western blot. Conversely, FAT10 overexpression resulted in increased PFKFB3 expression. (B, C) qRT-PCR and western blot analysis of PFKFB3 expression in 30 OS tissues and their corresponding adjacent tissues. The level of PFKFB3 mRNA in OS samples was about twice that in the corresponding adjacent tissues (B). PFKFB3 protein expression increased in 25 OS cases, while no significant differences compared to controls were detected in the remaining 5 cases (C). (D) Immunohistochemistry (IHC) results showed that the expression level of PFKFB3 protein was significantly higher in OS tissues compared to normal adjacent tissues (** $P < 0.01$). (E, F) Scatter plots of FAT10 and PFKFB3 mRNA (E) and protein (F) expression in OS tissues (** $P < 0.01$).

and HA-FAT10 plasmids and added the proteasome inhibitor MG132. The results showed that when OS cells were treated with MG132, FAT10 expression had no significant impact on EGFR levels (Figure 8C). In addition, degradation kinetics experiments showed that the half-life of EGFR in FAT10-overexpressing OS cells was significantly longer than that in control cells (Figure 8D). These results indicated that FAT10 regulated EGFR degradation. Finally, in order to clarify the mechanism of EGFR regulation by FAT10, we treated OS cells with MG132 after transfection with shFAT10 and HA-FAT10 plasmids. Immunoprecipitation experiments using an anti-EGFR antibody showed that FAT10

knockout and overexpression increased and reduced the level of EGFR ubiquitination, respectively (Figure 8E). These results suggested that FAT10 stabilizes EGFR by controlling its ubiquitination.

Discussion

Despite recent progress in OS prognosis and treatment methods, the 5-year survival rate has not substantially improved [29]. Therefore, an in-depth biological understanding of OS pathogenesis is important. In recent years, the relevance of metabolic reorganization in the occurrence and progression of tumors has

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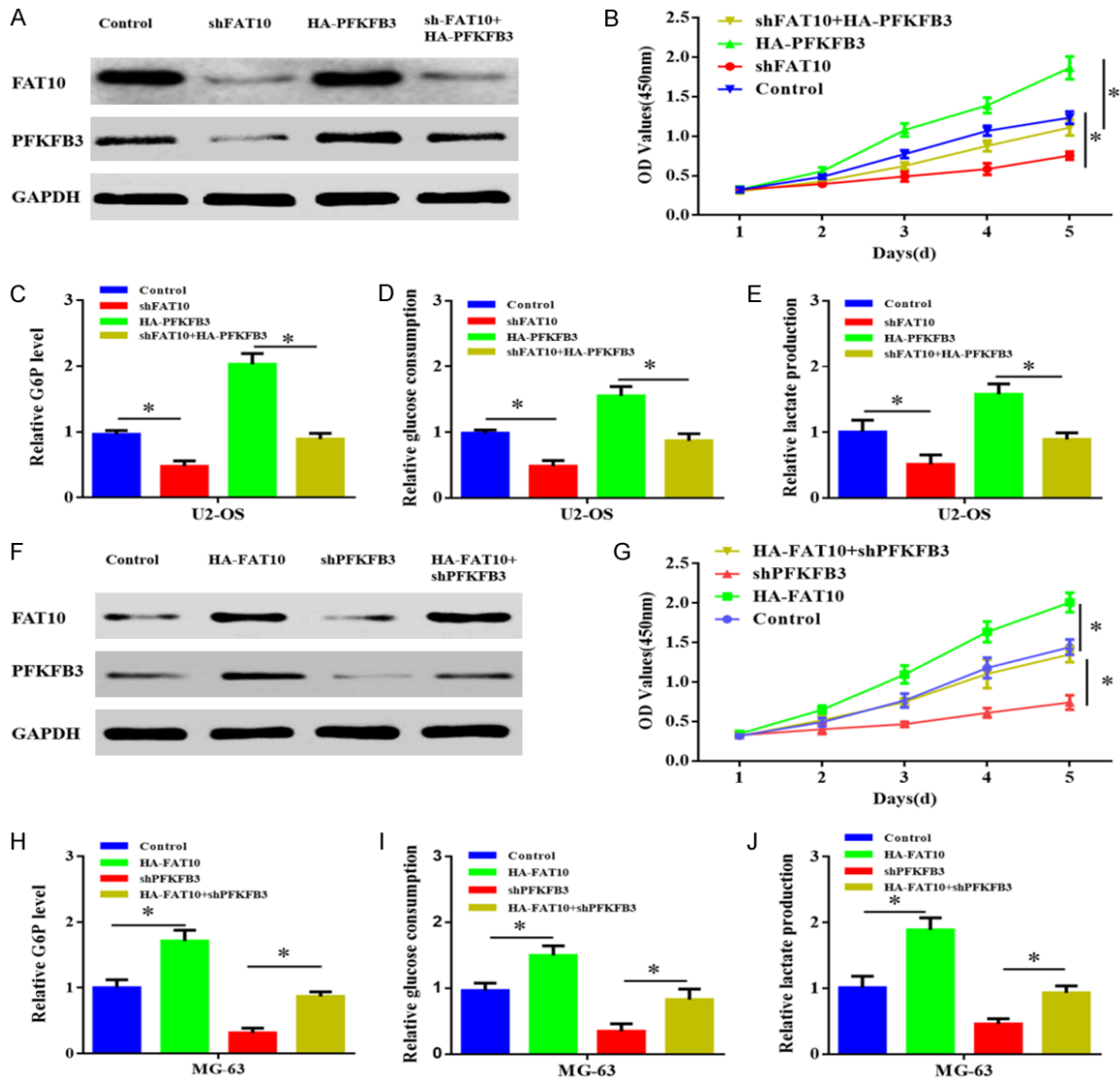


Figure 6. PFKFB3 mediates FAT10-induced glycolysis and proliferation in osteosarcoma (OS) cells. (A) Western blotting of FAT10 and PFKFB3 in U2-OS cells stably transfected with shFAT10 in the presence or absence of HA-PFKFB3. (B) CCK8 assays showed that the reduced proliferation induced by FAT10 knockdown in U2-OS cells was partially abolished by the introduction of HA-PFKFB3 (* $P < 0.05$). (C-E) Cellular G6P levels (C), glucose consumption (D), lactate production (E) showed that the reduced induced by FAT10 knockdown in U2-OS cells was partially abolished by the introduction of HA-PFKFB3. (F) Western blotting of FAT10 and PFKFB3 in MG-63 cells stably transfected with HA-FAT10 in the presence or absence of shPFKFB3. (G) CCK8 assays showed that the increased proliferation induced by FAT10 overexpression in MG-63 cells was partially abolished by the introduction of shPFKFB3 (* $P < 0.05$). (H-J) The increase in cellular G6P levels (C), glucose consumption (D), and lactate production (E) induced by FAT10 overexpression in MG-63 cells was partially abolished by transfection with shPFKFB3.

been widely demonstrated, and this phenomenon is considered a hallmark of cancer [30]. OS has an extremely high degree of malignancy. Because of its differing dependence on metabolism, prognosis is extremely poor. Therefore, in order to provide a new theoretical basis for OS treatment, it is necessary to explore this disease from a metabolic perspective. This study demonstrated that high FAT10 expres-

sion predicts poor OS prognosis, and that FAT10 plays a key role in regulating glycolysis.

FAT10 is a ubiquitin-like protein, and its high expression in liver cancer [9], breast cancer [31], and colorectal cancer has been demonstrated [10]. Moreover, it has been shown to promote the pathogenesis of several human cancers. Evidence indicates that FAT10 regu-

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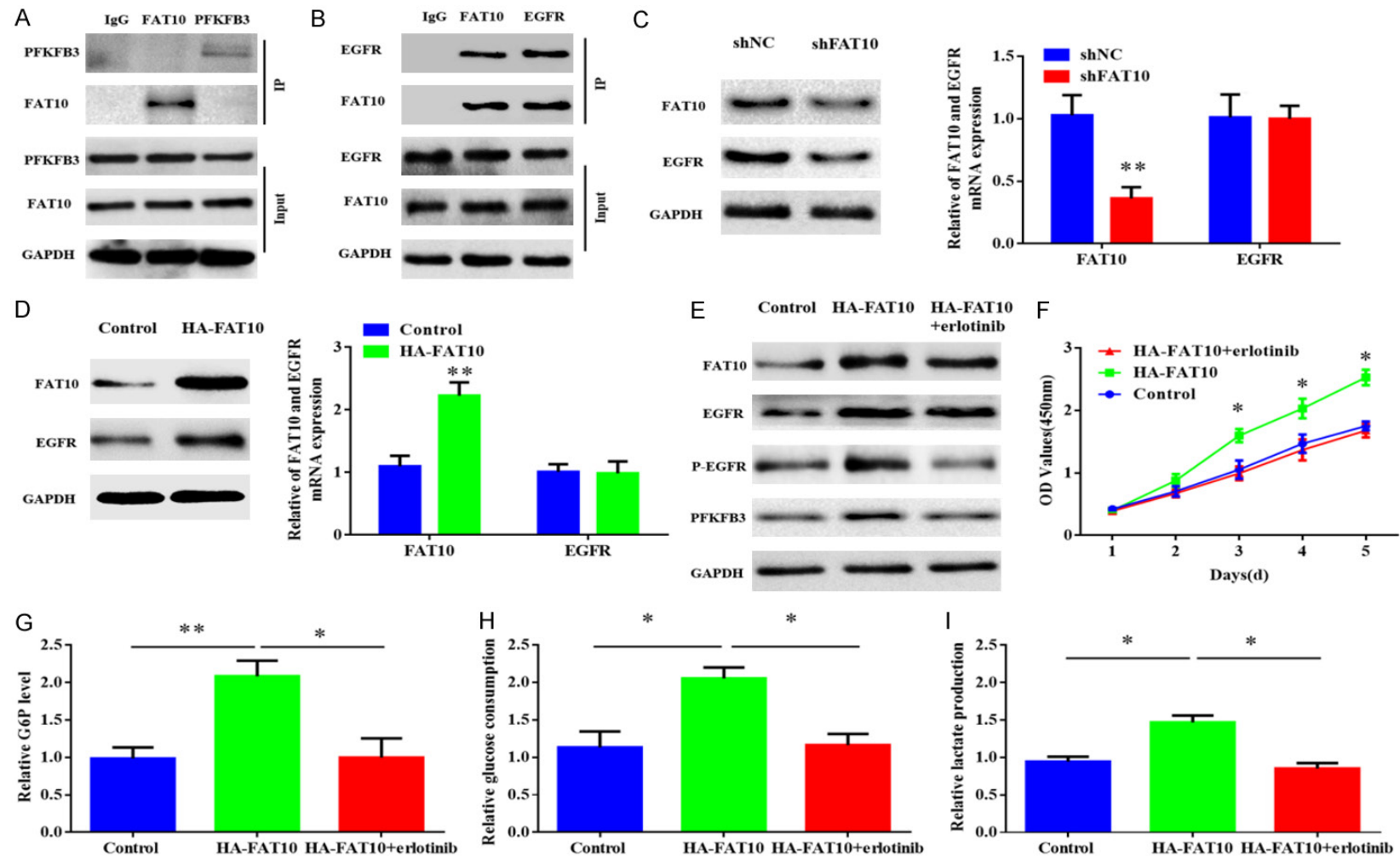


Figure 7. FAT10 regulates PFKFB3 expression by activating EGFR in osteosarcoma (OS) cells. (A) Co-IP results showing that FAT10 and PFKFB3 did not directly interact. (B) Co-IP results indicating that FAT10 directly bound to EGFR in OS cells. (C, D) FAT10 downregulation in OS cells decreased the level of EGFR protein. Conversely, FAT10 overexpression increased EGFR expression, while the level of EGFR mRNA remained unchanged (** $P < 0.01$). (E) PFKFB3 and P-EGFR protein levels in FAT10-overexpressing MG-63 cells treated with the EGFR inhibitor (erlotinib). (F) CCK-8 assays to assess the proliferation of HA-FAT10-MG-63 cells treated with the EGFR inhibitor (erlotinib). (G-I) Cellular G6P levels (G), glucose consumption (H), and lactate production (I), in OS cells stably transfected with the indicated plasmid or treated with the EGFR inhibitor (erlotinib) (*** $P < 0.001$, ** $P < 0.01$, * $P < 0.05$).

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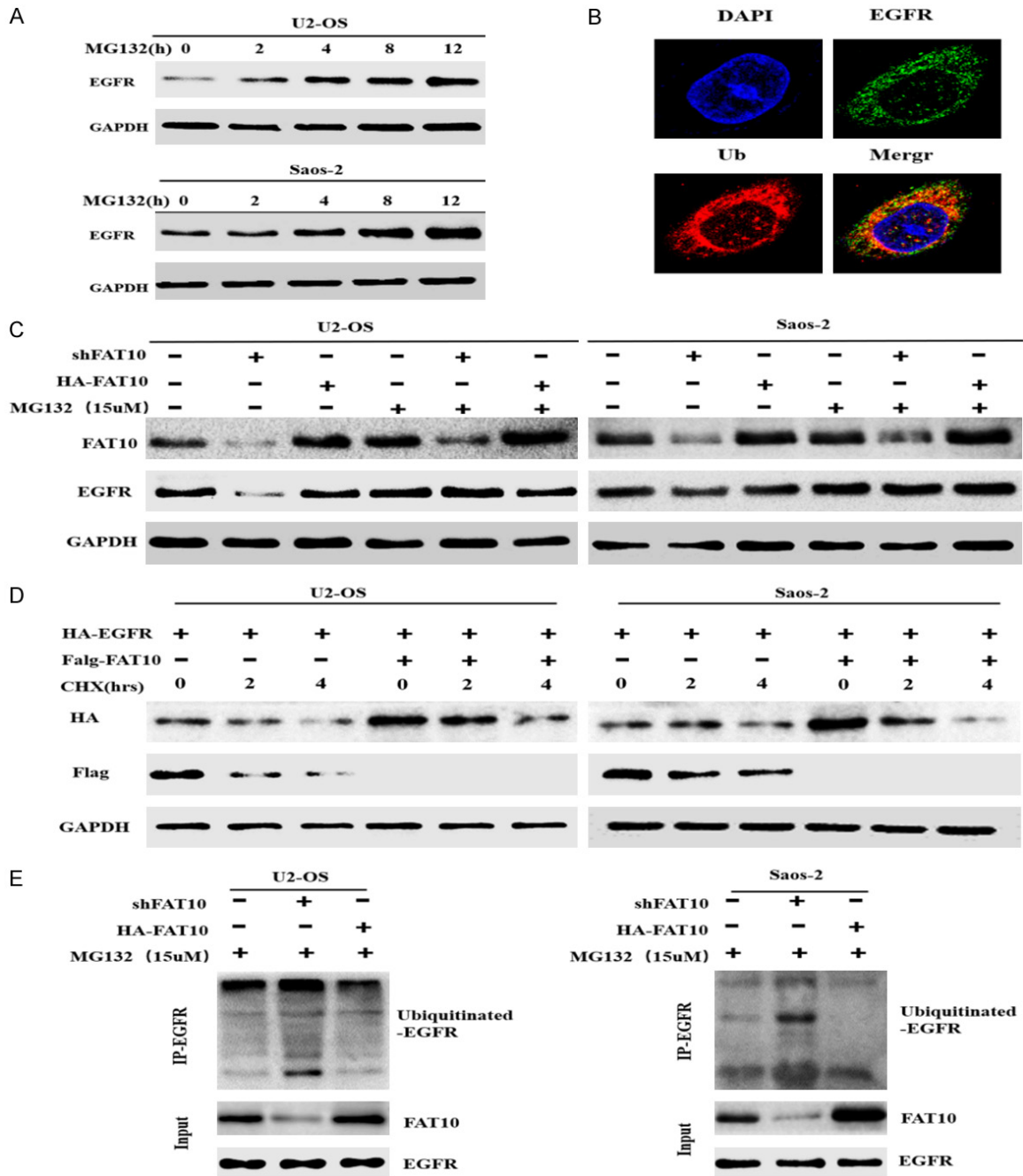


Figure 8. FAT10 stabilizes EGFR by regulating EGFR ubiquitination in osteosarcoma (OS) cells. **A.** OS cells were treated with the protease inhibitor MG132 (15 μ M) for a specific period of time, and EGFR expression was examined by western blot. **B.** Co-localization of EGFR and Ub in OS cells. **C.** FAT10 does not affect EGFR expression, as assessed in OS cells transfected with shFAT10/HA-FAT10 plasmid and treated with MG132. **D.** Detection of EGFR degradation by using anti-HA and anti-Flag antibodies in U2-OS and Saos-2 cells transfected with the HA-EGFR expression plasmid, with or without the Flag-FAT10 plasmid, and treated with cycloheximide for the indicated times. **E.** Lysates from OS cells transduced with shFAT10/HA-FAT10 were treated with MG132 (15 μ M) for 4 h, collected, immunoprecipitated with an anti-Ubiquitin (Ub) antibody, and then immunoblotted with an EGFR antibody.

lates cell adhesion, contraction, proliferation, migration, and apoptosis [32]. Recent studies have shown that FAT10 regulates cancer pro-

gression. Yan et al. found that FAT10 influences WISP1 mRNA and protein expression, thus promoting the progression of hepatocellular carci-

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Figure 9. Proposed model by which the ubiquitin-like protein FAT10 promotes osteosarcoma growth and glycolysis. FAT10 upregulates PFKFB3 by increasing the stability of EGFR and suppressing its ubiquitination.

noma [33]. FAT10 promotes breast cancer metastasis by stabilizing ZEB2 expression [31]. Consistently, we previously reported that FAT10 promotes tumor growth by stabilizing YAP1 expression [28]. The current study demonstrated that FAT10 was highly expressed in OS tissues and cells, and that its expression level was closely related to tumor size, TNM stage, and prognosis. Moreover, FAT10 promoted OS growth both *in vivo* and *in vivo*. Our findings suggested that FAT10 may represent a novel indicator of poor prognosis in OS patients and function as an oncogene in OS progression.

Metabolic changes in tumor cells help them survive and grow in harsh microenvironments. Therefore, a deeper understanding of the regulation of aerobic glycolysis in OS may help provide a more robust theoretical basis for the development of new targeted therapies. Recent studies have emphasized the importance of glucose metabolism reprogramming for OS cell growth. For example, miR-185 inhibits glycolysis by targeting hexokinase 2 (HK2), and promotes the adaptation of OS cells to metabolic stress [34]. Here, we showed that FAT10 affected glucose metabolism in OS cells. Specifically, FAT10 promoted aerobic glycolysis. These findings provide insight into the biological function of FAT10 in cancer, and demonstrate its potential as a novel therapeutic target.

In order to study the impact of FAT10 on aerobic glycolysis, we focused on PFKFB3, a key and highly active glycolytic enzyme. Its increase was previously found to promote PFK-1 activity, thereby enhancing glucose uptake by tumor

cells and rapid glycolysis, and providing sufficient energy for tumor growth [35]. Moreover, PFKFB3 is very important for the growth of tumors containing Ras-transformed cells, and promotes angiogenesis and tumor growth [36]. Previous studies have shown that the activation of the HIF-1 α [35] and p38/MK2 signaling pathways [37], as well as PFKFB3 phosphorylation at serine 461 [38], upregulate its expression and promote tumor proliferation. In the present study, a new mechanism promoting OS glycolysis and proliferation was addressed. This mainly consisted of FAT10

upregulation and the subsequent increase in PFKFB3 expression. First, we showed that the expression levels of FAT10 and PFKFB3 were increased and positively correlated in OS tissues. In addition, FAT10 downregulation in OS cells caused a decrease in PFKFB3 expression, as well as a significant reduction of tumor growth and the glycolytic rate. PFKFB3 upregulation restored proliferation and increased the rate of glycolysis in OS cells, while PFKFB3 silencing attenuated FAT10-induced OS cell proliferation and glycolysis. Collectively, these results demonstrated that FAT10 affected OS progression by regulating PFKFB3 expression. Next, the mechanism behind this event was explored. Previous studies have shown that FAT10 is the only ubiquitin-like protein that does not depend on ubiquitin, and directly induces substrate degradation via the proteasome [39]. Under the action of E1 and E2 enzymes, FAT10 binds to a Lys residue at the carboxyl end of the substrate protein, is recognized by the receptor subunit on the proteasome, and is finally degraded [40]. However, we found that FAT10 did not directly interact with PFKFB3. It has been demonstrated that EGFR plays a key role in regulating cancer progression [41]. Moreover, EGFR is involved in the regulation of tumor glycolysis [24]. In addition, the Lypova study reported that EGFR regulates PFKFB3 expression [23]. Here, we unveil a novel mechanism by which FAT10 regulates PFKFB3 expression by affecting EGFR expression. This conclusion is based on the following observations. First, we found that FAT10 downregulation decreased EGFR expression, while FAT10 overexpression yielded the opposite

effect. Furthermore, FAT10 directly bound to EGFR. Inhibition of EGFR activity in FAT10-overexpressing OS cells prevented PFKFB3 upregulation and the increase of cell proliferation and glycolysis levels. Taken together, these data demonstrated that FAT10 regulation of PFKFB3-induced OS cell proliferation and aerobic glycolysis depended on EGFR.

Finally, we further explored the impact of FAT10 on EGFR activity. Previous research has shown that FAT10 stabilizes substrate proteins such as YAP1 [28] and β -catenin [9], preventing their degradation, and that EGFR can be degraded by the UPS. Thus, we reasoned that FAT10 could stabilize EGFR expression by affecting its ubiquitination. First, FAT10 directly bound to EGFR. Second, FAT10 knockdown promoted EGFR ubiquitination, whereas FAT10 overexpression significantly reduced EGFR ubiquitination. In conclusion, we provided evidence that FAT10 was upregulated in OS tissues and associated with OS progression in patients. Moreover, FAT10 promoted the growth and aerobic glycolysis of OS cells. More importantly, FAT10-induced aerobic glycolysis was dependent on PFKFB3 in pancreatic cells. Our findings also demonstrated that FAT10 regulation of PFKFB3 expression depended on a direct interaction between FAT10 and EGFR, which affected EGFR ubiquitination and degradation (**Figure 9**). On the basis of these findings, we conclude that FAT10 is a candidate biomarker for OS diagnosis and a novel potential therapeutic target.

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Disclosure of conflict of interest

None.

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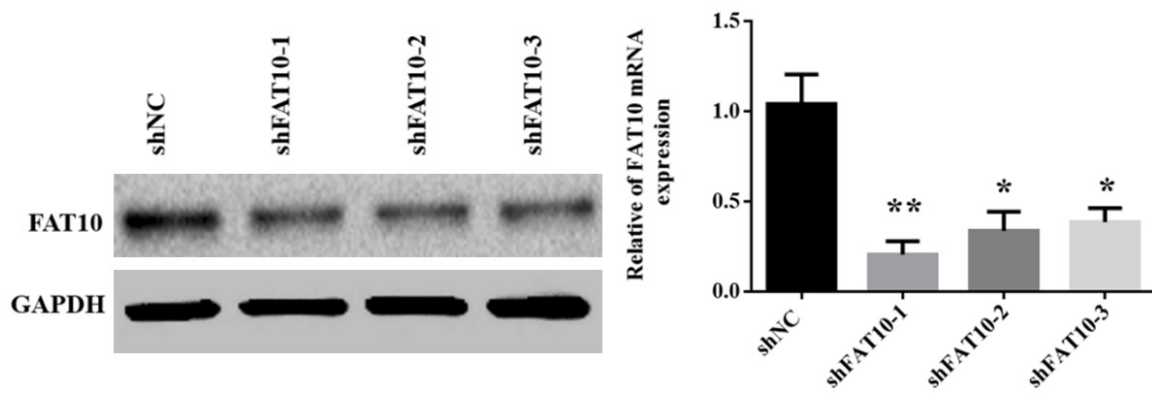


Figure S1. FAT10 expression decreased after transfection of the corresponding shRNA plasmids. Efficiency of FAT10 silencing in OS cell lines was measured by qRT-PCR and western blot.