Cancer Letters 448 (2019) 105–116

Contents lists available at ScienceDirect

Cancer Letters

journal homepage: www.elsevier.com/locate/canlet

Original Articles

ZBTB7A, a miR-663a target gene, protects osteosarcoma from endoplasmic reticulum stress-induced apoptosis by suppressing LncRNA GAS5 expression

Lu Zhang[a,1], Yuan Wang[a,1], Li Zhang[b,1], Xin Xia[a,1], Yulin Chao[a], Ruiping He[a], Chuanchun Han[a,*], Wenzhi Zhao[a,**]

[a] The Second Affiliated Hospital & Institute of Cancer Stem Cell, Dalian Medical University, Dalian, Liaoning, 116044, PR China
[b] Laboratory of Pathogenic Biology, College of Basic Medical Science, Dalian Medical University, Dalian, 116044, China

ARTICLE INFO

Keywords:
ZBTB7A
Osteosarcoma
GAS5
miR-663a
ER stress

ABSTRACT

Many studies have uncovered the essential role of ZBTB7A in regulating tumourigenesis. However, its functional significance in cell responses to endoplasmic reticulum stress (ER stress) remains poorly understood. Here we report that ZBTB7A functions as an important prosurvival factor in osteosarcoma cells undergoing pharmacological ER stress-induced by tunicamycin (TM) or thapsigargin (TG). The downregulation of ZBTB7A expression by ER stress promoted cell apoptosis *in vitro* and *in vivo*. ZBTB7A expression levels were increased in osteosarcoma tissues and elevated ZBTB7A was associated with osteosarcoma metastasis. Further mechanistic studies revealed that miR-663a induced by ER stress directly bound to the 3′UTR of ZBTB7A and contributed to ER stress-induced ZBTB7A downregulation in osteosarcoma cells. Additionally, our data revealed that ZBTB7A bound to the promoter of LncRNA GAS5 and transcriptionally suppressed LncRNA GAS5 expression, leading to a decline in ER stress-induced cell apoptosis. Collectively, our findings reveal the prosurvival role of ZBTB7A in osteosarcoma adaptation to ER stress and suggest that the miR-663a-ZBTB7A-LncRNAGAS5 pathway is essential for the survival of human osteosarcoma cells under ER stress.

1. Introduction

Osteosarcoma is the most commonly occurring primary malignant bone tumour that most frequently develops in childhood and adolescence. In recent years, the 5-year survival rates of patients with localized osteosarcoma have remained at 60–70% due to surgery together with multi-agent chemotherapy [1]. However, the 5-year survival rate of patients with metastatic osteosarcoma is less than 20% [2,3]. As is the case for other cancers, distant metastasis remains a major obstacle to the treatment of osteosarcoma.

Cancer cells are able to spread from the tumour of origin to colonize distant organs where they can form another tumour [4]. When tumours metastasise, cells are subjected to diverse microenvironments such as hypoxia and nutrient starvation, leading to the accumulation of improperly folded proteins in the endoplasmic reticulum (ER) lumen, resulting in ER stress [5]. When this occurs, an adaptive mechanism called the unfolded protein response (UPR) is triggered to help the cell cope with this change and restore protein homeostasis in the ER, later contributing to tumourigenesis and metastasis [6]. Therefore, understanding potential molecular mechanisms of adaptation to ER stress is beneficial in developing new strategies for preventing tumour metastasis and for improving the survival rates of patients with metastatic osteosarcoma.

ZBTB7A, also known as Pokemon, LRF, or FBI, is a member of the POK family of transcriptional repressors, which consists of an NH2-terminal POZ/BTB domain and 4 COOH-terminal krüppel-type zinc fingers. The POZ/BTB domain is involved in homodimerization or heterodimerization and recruits corepressors such as BcoR, NcoR, or SMRT while the krüppel-type zinc finger domain mediates specific forms of DNA recognition and binding [7,8]. ZBTB7A has been reported to proliferate in some human cancers such as breast cancer, colorectal cancer, prostate cancer and bladder cancer and to play an important role in tumourigenesis [9,10]. However, some studies have indicated that ZBTB7A acts as a tumour suppressor by repressing glycolysis and metastasis [11,12]. Our finding indicate that ZBTB7A enhances osteosarcoma chemoresistance by suppressing LINC00473 expression [13]. Although different functions have been reported, the role of ZBTB7A in osteosarcoma adaptation to ER stress remains unclear.

* Corresponding author.
** Corresponding author.
E-mail addresses: hanchuanchun@163.com (C. Han), drzwz@163.com (W. Zhao).
[1] The authors contributed equally to this work.

https://doi.org/10.1016/j.canlet.2019.01.046
Received 14 October 2018; Received in revised form 26 January 2019; Accepted 29 January 2019

In this study, we found that ZBTB7A plays an essential role in osteosarcoma adaptation to ER stress. ZBTB7A decline induced by ER stress promoted cell apoptosis *in vitro* and in *vivo*. Further mechanistic studies revealed that miR-663a induced under ER stress directly bound to the 3′UTR of ZBTB7A and contributed to ZBTB7A downregulation under ER stress. Additionally, our data revealed that ZBTB7A transcriptionally suppressed GAS5 expression under ER stress, leading to a decline in cell apoptosis. Collectively, our findings reveal the pro-survival role of ZBTB7A in osteosarcoma adaptation to ER stress and suggest that the miR-663a-ZBTB7A-GAS5 pathway is essential for the survival of human osteosarcoma cells under ER stress.

2. Methods

2.1. Cell culture and reagents

U2OS and 143B cells were maintained in Dulbecco's modified Eagle medium. MG63 cells were cultured in Eagle's Minimum Essential Medium (EMEM). Saos-2 cells were cultured in McCoy's5A Medium. These media were supplemented with 10% foetal bovine serum (FBS) (ExCell Bio, Lot: FSP500), 2 mM L-glutamine, penicillin (100 U/ml), streptomycin (100 μg/ml) and 0.1% Savelt ™ (Hanbio Co. LTD 1:1000) in a humidified atmosphere of 5% CO_2 maintained at 37 °C. The following antibodies were used in this study: antibodies against GAPDH (Santa Cruz Biotechnology, Dallas, TX, USA; SC-25778, 1:1000), PARP (Santa Cruz Biotechnology, SC-8007, 1:1000), ZBTB7A (Santa Cruz Biotechnology, SC-33683, 1:500 for ChIP, 1:1000 for WB), and GRP78 (Santa Cruz Biotechnology, SC-13968, 1:1000 for WB). Tunicamycin (TM, Lot:T7765) and thapsigargin (TG, Lot:T9033) were purchased from Sigma Chemical Co. They were dissolved in DMSO and developed in stock solutions of 3 mmol/L for tunicamycin and of 1 mmol/L for thapsigargin. The cells were treated with 3 μmol/L TM or 1 μmol/L TG at the indicated times.

2.2. Plasmids and transfection

ZBTB7A was constructed into pCDNA3.0-Flag vector. We transfected 5 μg plasmids into osteosarcoma cells (1×10^6) using Lipofectamine 3000 (Invitrogen, Carlsbad, CA, USA) according to the Lipofectamine™ 3000 Reagent Protocol. Empty vectors were then used as the negative control. The lentiviral expression vector for ZBTB7A and GAS5 was constructed by inserting full-length cDNA into a pCDH vector. To generate a lentivirus expressing ZBTB7A or GAS5, HEK 293T cells grown on a 6-cm dish were transfected with 2 μg pCDH-ZBTB7A or GAS5 and control vector (pCDH), 1.5 μg psPax2, and 0.5 μg pMD2G. At 24 h after transfection, cells were cultured with DMEM containing 10% FBS for an additional 24 h. The culture medium containing lentiviral particles was centrifuged at 1000 g for 5 min. Viral particles collected in the supernatant were used for infection. To establish a stable cell line, the puromycin (2.5 μg/ml) was used as a selection marker for the infected cells. The expression efficiency was evaluated by western blot analysis.

2.3. RNA interference

RNA interference was performed as previously described [14,15]. In brief, to generate ZBTB7 stably knockdown cells, the ZBTB7A shRNA sequences were cloned into the pLKO-puro vector. The targeting sequences of ZBTB7A were as follows: No.1 5-CCACTGAGACACAAACC TATT-3 and No.2 5-GAACGTGTACGAGATCGACTT-3. pLKO.1-shRNAZBTB7A or pLKO.1 vector, pVSVG, pREV and pGAG were co-transfected into HEK293T cells for 24hrs, and cell culture media were collected. The viruses were used to infect cells in the presence of polybrene. Forty-eight hours later, osteosarcoma cells were cultured in medium containing 2.5 mg/ml puromycin for the selection of stable clones. The knockdown efficiency was evaluated by western blot analysis.

LncRNA GAS5 was knocked down in osteosarcoma cell using the siRNAs. The siRNA sequences for GAS5 knockdown were as follows: No.1 5-GGACCAGCTTAATGGTTCT-3 and No.2 5-GCAAGCCTAACTCA AGCCA-3. The knockdown efficiency was evaluated by q-RT-PCR analysis.

2.4. Introduction of microRNA mimics and inhibitors

Mimics and inhibitors of miRNA-663a were synthesized by the GenePharma Company (Shanghai, People's Republic of China). For each transfection in a six-well plate, 100 nM miRNA mimics, scrambles or inhibitors were used. The transfection of osteosarcoma cells by Oligofectamine (Invitrogen) was performed according to the manufacturer's instructions.

2.5. Real-time RT-PCR and RT-PCR

Total RNA was isolated using Trizol (Invitrogen). One microgram of total RNA was used to synthesize cDNA using the PrimeScriptTM RT reagent kit (Takara, RR047A) according to the manufacturer's instructions. The following primers were used: Actin: F: 5-GACCTGACTGAC TACCTCATGAAGAT-3 and R:5-GTCACACTTCATGATGGAGTTGAAGG-3; ZBTB7A: F:5- CCCTACGAGTGCAACATCTG-3 and R:5- CTTCAGGTC GTAGTTGTGGG-3; and GAS5: F: 5- ACAGGCATTAGACAGAAAGC-3 and R: 5- TACCCAAGCAAGTCATCCA-3. ZBTB7A and GAS5 levels were normalized to those of β-actin, and miR-663a levels were normalized to U6. Changes in gene expression were determined using the $2^{-\Delta\Delta CT}$ method. Primers for mature miR-663a were purchased from Takara.

2.6. Tissue microarrays and immunohistochemistry

Osteosarcoma tissue microarrays were purchased from Alenabio (Xi'an, China), and they contained a total of 42 osteosarcoma tissues and 22 normal tissues. Immunohistochemistry was performed as previously described [16]. Characteristics of the patients and their tumours were collected through a review of medical records and pathologic reports. Informed consent with approval of the ethics committee of Taizhou Hospital of Zhejiang Province was obtained. All methods used for this study were in accordance with the approved guidelines, and all experimental protocols employed were approved by the ethics committee of Taizhou Hospital of Zhejiang Province.

Sections were stained with Masson's trichrome and H&E (haematoxylin and eosin) for histopathological examination. For immunohistochemistry, sections were subjected to antigen retrieval using microwave heating at 95 °C in citrate buffer (pH = 6.0, for ZBTB7A). The indicated antibodies specific to ZBTB7A (1:200) were diluted according to the manufacturer's instructions. Degrees of immunostaining were reviewed and scored by two independent observers. The proportion of the stained cells and the extent of the staining were used as criteria of evaluation. For each case, at least 1000 tumour cells were analysed. For each sample, the proportion of ZBTB7A expressing cells varied from 0% to 100%, and the intensity of staining varied from weak to strong. One score was given according to the percentage of positive cells as follows: < 5% of the cells: 1 point; 6–35% of the cells: 2 points; 36–70% of the cells: 3 points; > 70% of the cells: 4 points. Another score was given according to the intensity of staining as follows: negative staining: 1 point; weak staining (light yellow): 2 points; moderate staining (yellowish brown): 3 points; and strong staining (brown): 4 points. A final score was then calculated by multiplying the above two scores. When the final score was equal to or greater than four, protein expression in the tumour was considered high; otherwise, protein expression in the tumour was considered low.

2.7. ChIP assay

The ChIP assay was performed as previously described [13]. In brief, MG63 cells treated with or without 1 μM TG or 3 μM TM for 36h and then the cells were cross-linked with 1% formaldehyde for 10 min at room temperature. ChIP assay was performed according to the manufacturer's instructions by using anti-ZBTB7A, and the ChIP assay kit (Millipore, Merck KGaA, Darmstadt, Germany). Anti-mouse IgG was used as control. The bound DNA fragments were eluted and amplified by PCR using the following primer pair: 5- TAAGTGTTAAC TATAAG-3 and 5- CAGCCATTTTTTAAAA-3. PCR products were separated on 2% agarose gel by gel electrophoresis.

2.8. Promoter reporters and dual-luciferase assay

The promoter of GAS5 and the matching mutant were constructed into a pGL3-basic vector. 3′UTR of ZBTB7A was constructed into a pSICHECK2 vector. Luciferase activity was measured in a 1.5-ml Eppendorf tube with a Promega Dual-Luciferases Reporter Assay kit (Promega E1980) according to the manufacturer's protocol after transfection. Relative Renilla luciferase activity was normalized to firefly luciferase activity. The assay was performed as previously described [17,18].

2.9. Long non-coding RNAs and miRNA sequencing analysis

U2OS cells were treated with 1 μM TG for the indicated times. Total RNA was isolated using Trizol reagent (Invitrogen, Carlsbad, CA, USA). Small RNA and long non-coding RNA sequencing were performed by KangChen Bio-tech using the Illumina Small RNA Sequencing Platform. The TruSeq small RNA library preparation kit (Illumina, San Diego, CA, USA) was used for library preparation. Sequencing was performed using an Illumina HiSeq 2000 sequencing system, and 10 Mb of clean reads were analysed using routine algorithms (KangChen Bio-tech, Inc., Shanghai, China).

2.10. Tumour growth and metastasis assay

Animal studies were conducted in accordance with the National Institute of Health Guide for the Care and Use of Laboratory Animals with the approval of the Animal Research Committee of Dalian Medical University. Male nude mice (4–6 weeks of age, 18–20 g) were obtained from the SPF Laboratory Animal Center of Dalian Medical University (Dalian, China) and were randomly divided into the indicated groups. Mice (n = 6) in the groups were subcutaneously injected with 1×10^6 143B cells with or without ZBTB7A knockdown. The 143B cells containing the pLKO.1vector were used as the negative control and the first sequence (No.1) of shRNA ZBTB7A was used for the *in vivo* mice experiment. These stable cells were maintained in Dulbecco's modified Eagle medium containing 2.5 mg/ml puromycin for the selection of stable clones. After 12 days, tumour size was measured every 4 days with Vernier callipers and converted to TV according to the following formula: TV (mm^3) = (axb^2)/2 where a and b are the largest and smallest diameters, respectively. All animals were killed 4 weeks after injection, and the transplanted tumours were removed, weighed and fixed for further study.

2.11. Caspase-3 activity assay and cell viability assay

Caspase-3 activity was analysed using the Caspase-3 Activity Assay Kit (Roche) according to the manufacturer's instructions. In brief, the tissue lysate was incubated with 100 μL of caspase-3 reagent at 37 °C for 1–2 h, and then, the fluorescence intensity (at 370–425 nm excitation and 490–530 nm emission wavelengths) was measured using a Spectra MAX M5 spectrophotometer (Molecular Devices).

Osteosarcoma cells were plated in 96-well plates at a density of 1000 cells in 200 μl of medium per well for 24 h and then were treated with 1 μM TG or 3 μM TM as indicated. Cell viability was determined using the CCK-8 kit (Cell Counting Kit-8).

2.12. Annexin V-FITC staining and TNUEL assay

The staining protocol was performed following the manufacturer's instructions (BD). Generally, Osteosarcoma cells (5×10^5) treated as indicated were harvested by a 5 min centrifugation at 1000*g* and resuspended in 195 μL binding buffer, followed by a 10 min incubation with 5 μL Annexin V-FITC at room temperature avoiding any light. After an additional centrifugation, the cells were resuspended in 190 μL binding buffer and 10 μL PI stain was added with slight shaking. FACS (BD) analysis was employed for detecting cell apoptotic events.

Osteosarcoma cells treated as indicated were analysed by the TUNEL Apoptosis detection kit (FITC) (40306ES20) and the protocol was performed following the manufacturer's instructions.

2.13. Statistics and data analyses

The data are expressed as mean ± SD values and a statistical evaluation was performed using a one-way analysis of variance (ANOVA). Values of $p < 0.05$ were considered statistically significant.

3. Results

3.1. ZBTB7A inhibits ER stress-induced osteosarcoma cell apoptosis

To investigate the role of ZBTB7A in osteosarcoma adaptation to ER stress, we first examined the expression of ZBTB7A in U2OS and 143B cells in response to 1 μM thapsigargin (TG) or 3 μM tunicamycin (TM) to induce pharmacological ER stress. These cells were relatively sensitive to cell death induced by 1 μM TG or 3 μM TM and the activation of ER stress was evidenced by the induction of GRP78. Remarkably, the induction of ER stress downregulated ZBTB7A expression and facilitated cell apoptosis, as indicated by an increase in cleaved PARP in both U2OS and 143B cells (Fig. 1A and B and Figs. S1A–1B). However, the decrease in ZBTB7A induced by ER stress disappeared in ER stress-resistant osteosarcoma cells MG63 and Saos2, implying that ZBTB7A may be a regulator of ER stress-induced apoptosis (Fig. 1C and D and Figs. S1C–1D). To further confirm this conclusions, ZBTB7A was overexpressed in U2OS cells, and the cells were treated with 1 μM TG or 3 μM TM for the indicated times. Relative to the control cells, the elevation of ZBTB7A repressed ER stress-induced apoptosis and increased cell viability (Fig. 1E and H and Fig. S1E). On the other hand, the inhibition of ZBTB7A promoted cell apoptosis and decreased cell viability in MG63 and Saos2 cells (Fig. 1I–L and Fig. S1F).

3.2. Elevated ZBTB7A in osteosarcoma tissues promotes tumourigenesis in vivo

Based on the observation that ZBTB7A contributes osteosarcoma adaptation to ER stress *in vitro*, we examined whether ZBTB7A facilitates osteosarcoma cell growth and suppresses cell apoptosis *in vivo*. To prove this, 143B cells with or without ZBTB7A knockdown were injected into 4- to 6-week-old BALB/C (nu/nu) male nude mice. Compared to the control cells, the knockdown of ZBTB7A suppressed tumour growth as indicated by a decrease in tumour volume and tumour weight and accelerated cell apoptosis as indicated by Caspase 3 activity and increases in cleaved PARP (Fig. 2A and F and Fig. S2).

To further confirm our finding, we analysed the expression levels of ZBTB7A in osteosarcoma tissues by immunohistochemical staining. Compared to those of the normal tissues, the protein levels of ZBTB7A were markedly increased in osteosarcoma tissues. The increase in ZBTB7A was found to be positively correlated with osteosarcoma metastasis (Fig. 2G and I).

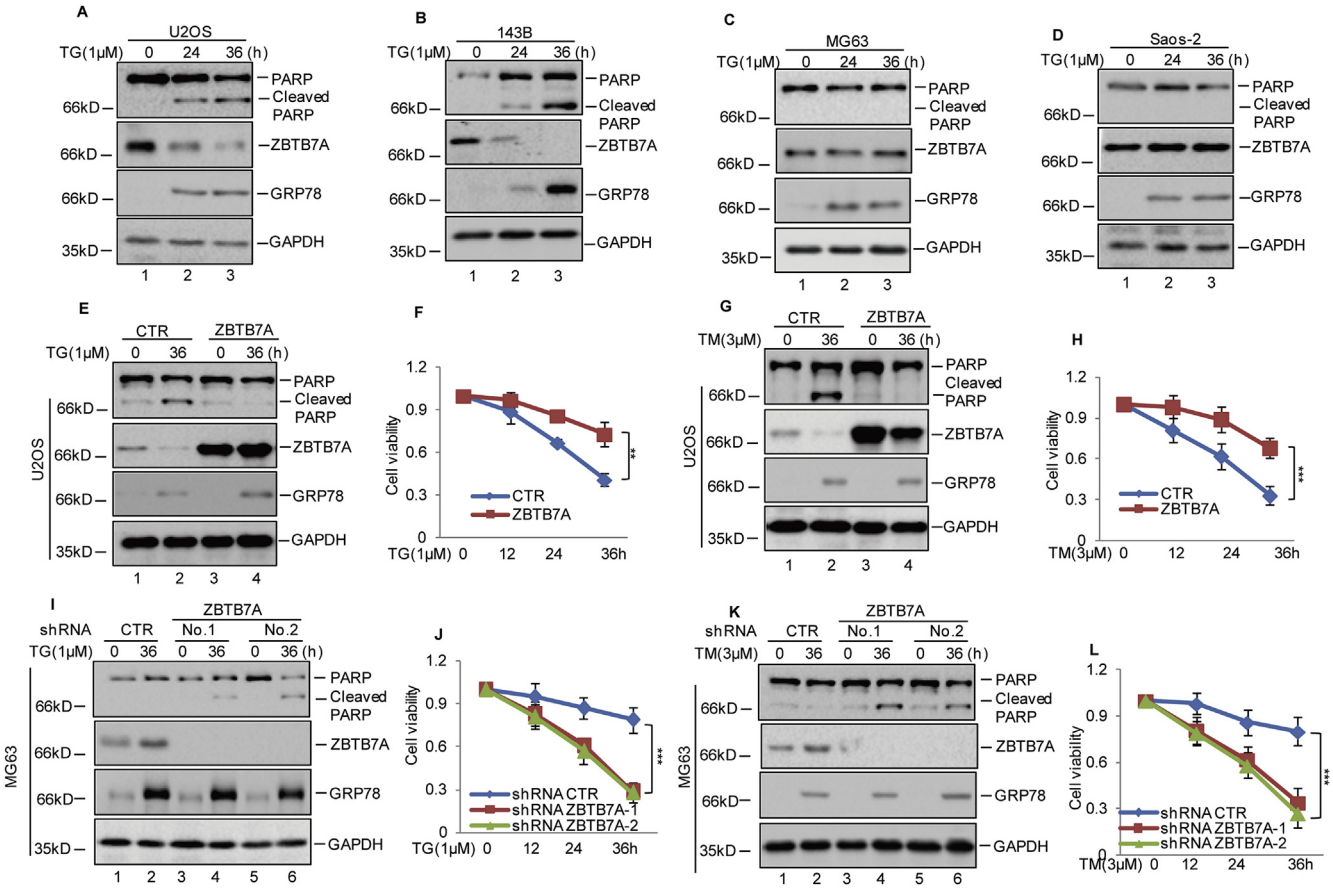


Fig. 1. ZBTB7A inhibited ER stress-induced osteosarcoma cell apoptosis. (A–D) osteosarcoma cell lines U2OS, 143B, MG63 and Saos2 were treated with 1 μM TG at the indicated times and cell lysates were then subjected to western blotting analysis using the indicated antibodies. (E–H) ZBTB7A was transfected into U2OS cells and then the cells were treated with 1 μM TG or 3 μM TM at the indicated times. Cell apoptosis and cell viability were measured by western blotting and CCK8 assays. The data represent mean ± SD values of the three independent experiments. **p < 0.01, ***p < 0.001 vs. control. (I–L) ZBTB7A was knocked down in MG63 cells and then the cells were treated with 1 μM TG or 3 μM TM at the indicated times. Cell apoptosis and cell viability were measured by western blotting and CCK8 assays. The data represent mean ± SD values of three independent experiments. ***p < 0.001 vs. control.

3.3. miR-663a inhibits ZBTB7A expression in osteosarcoma cells

To explore the mechanism underlying ER stress-induced ZBTB7A downregulation, we first analysed the mRNA levels of ZBTB7A in response to ER stress treatment and found that the mRNA levels of ZBTB7A were not altered in response to TG or TM treatment (Figs. S3A–3B). A large number of studies have shown that miRNAs are important regulators of ZBTB7A expression in a transcriptionally independent manner [19,20]. Thus, we aim to determine whether miRNAs are involved in regulating ZBTB7A expression under ER stress. To this end, different sequences of ZBTB7A 3′UTR were cloned by PCR and then were inserted into luciferase report pSicheck2 vector (Fig. 3A). Dual-luciferase report results show that the luciferase activity of ZBTB7A 3′UTR (P3) was significantly decreased in response to TG or TM treatment, indicating that the decline in ZBTB7 by ER stress resulted from miRNAs (Fig. 3B and C). Through a combined RNA sequencing analysis and web-based miRNA resources we found that miR-663a, miR-4787-5p, miR-1290, miR-448a and miR-6218, which were upregulated by ER stress, served as candidate miRNAs for ZBTB7A (Fig. 3D and E, Table S1 and Table S2). To verify this result, the miRNAs were transfected into U2OS cells together with ZBTB7A 3′UTR and luciferase activities were measured. As shown in Fig. 3F, miR-663a notably suppressed the activity of ZBTB7A 3′UTR(P3). To further confirm this finding, we constructed the 3′UTR region of ZBTB7A containing the wild type binding site (WT) or the corresponding binding mutant (Mut) for miR-663a in the luciferase reporter system and carried out luciferase assays (Fig. 3G). We found that the overexpression of miR-663a suppressed ZBTB7A expression and decreased luciferase activity, and this decrease was prevented when the binding site was mutated (Fig. 3H and K and Fig. S3C). Conversely, the inhibition of miR-663a increased ZBTB7A expression and upregulated luciferase activity (Fig. 3L and O and Fig. S3D). Thus, these data suggest that miR-663a inhibits ZBTB7A expression in osteosarcoma cells.

3.4. miR-663a contributes to ER stress-induced ZBTB7A downregulation and promotes cell apoptosis

Having identified that miR-663a can suppress ZBTB7A expression in osteosarcoma cells, we next explored whether miR-663a contributes to ER stress-induced ZBTB7A decline. To investigate this, we first confirmed the upregulation of miR-663a in response to ER stress and found the expression levels of miR-663a to be significantly increased in U2OS cells under ER stress (Fig. 4A). Subsequently, ZBTB7A 3′UTR was transfected into U2OS cells with or without miR-663a inhibitor and then the cells were treated with 1 μM TG or 3 μM TM to induce ER stress. We found that the decrease in luciferase activity and protein levels of ZBTB7A in response to TG or TM treatment disappeared when miR-663a inhibitor was overexpressed. Similar results were obtained for 143B cells (Fig. 4B and C and Figs. S4A–4D). On the other hand, the exogenous expression of miR-663a in MG63 cells inhibited luciferase activity and protein levels of ZBTB7A in response to ER stress (Fig. 4D and E and Figs. S4E–4F). Collectively, these data indicate that miR-663a contributed to ER stress-induced ZTBT7A decline.

Based data showing that ZBTB7A suppresses ER stress-induced

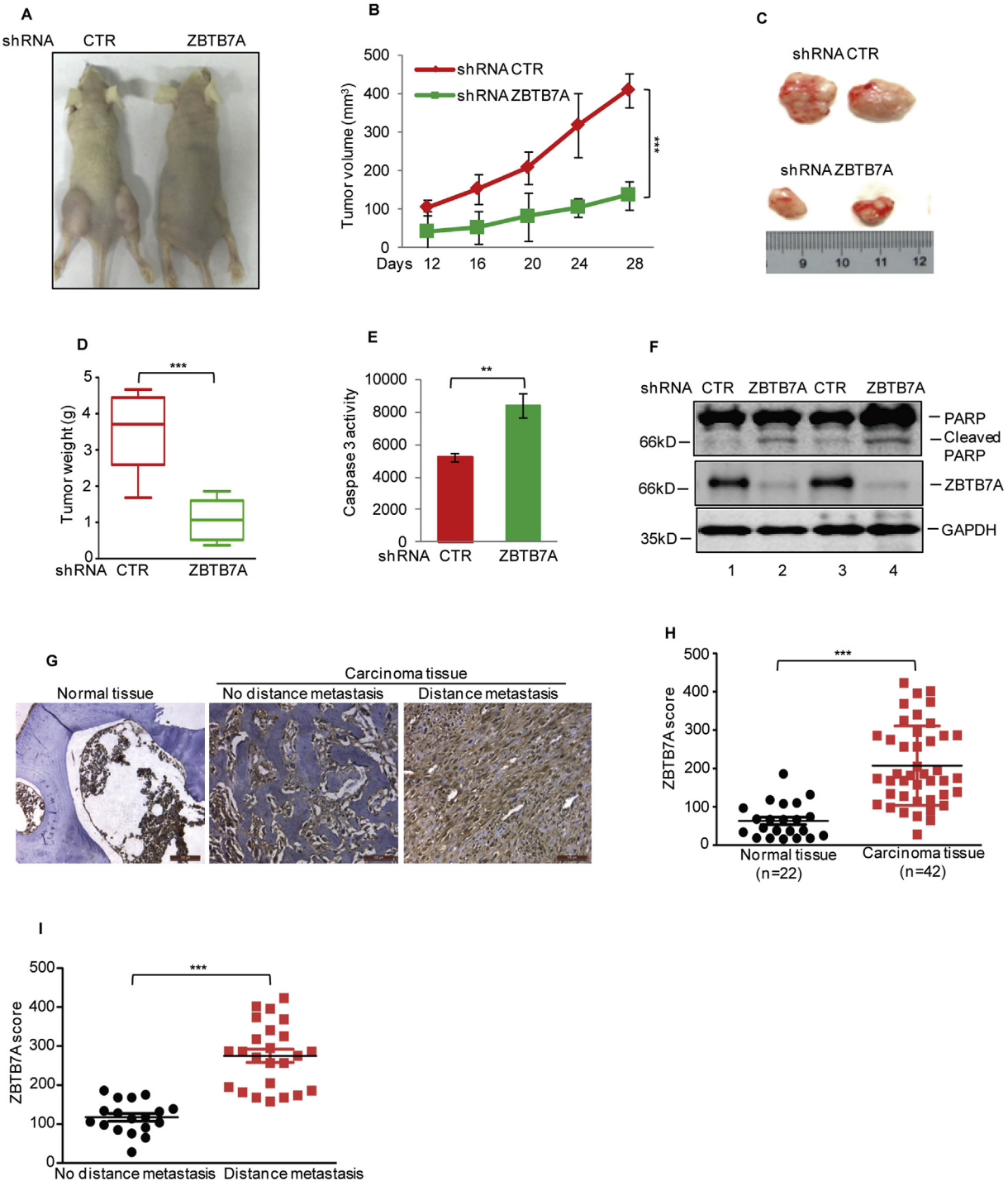


Fig. 2. ZBTB7A promotes tumourigenesis *in vivo* and is highly expressed in osteosarcoma tissues. (A–F) 143B cells with or without ZBTB7A knockdown were subcutaneously injected into nude mice (n = 6 in each group) for tumour formation (1×10^6 cells per mouse, 4 weeks). Representative bright-field imaging was taken of tumours in the mice implanted with the indicated cells. After 4 weeks, mice receiving transplants of indicated cells were sacrificed. Tumour volumes and weights were calculated. Caspase-3 activity was measured by a luciferase activity assay. The expression levels of cleaved PARP and ZBTB7A in tumours were measured via western blotting assay. $^{**}p < 0.01$, $^{***}p < 0.001$ vs. control. (G–H) The expression levels of ZBTB7A in 42 osteosarcoma tissues and 22 normal tissues were analysed by immunohistochemistry. Data represent the mean ± SD values of three independent experiments. $^{***}p < 0.001$ vs. control. (I) The correlation between ZBTB7A expression and distant metastasis was analysed in 42 patients with osteosarcoma. Data represent the mean ± SD values of three independent experiments. $^{***}p < 0.001$ vs. control.

apoptosis, we then aimed to investigate whether miR-663a regulates osteosarcoma adaptation to ER stress by targeting ZBTB7A. Thus, miR-663a inhibitor was introduced into U2OS cells with or without ZBTB7A knockdown and then the cells were treated with 1 μM TG or 3 μM TM. We found that the downregulation of miR-663a reduced ER stress-induced apoptosis, which disappeared when ZBTB7A was knocked down (Fig. 4F and Fig. S4G). Consistent with data drawn from knockdown studies, ZBTB7A overexpression reversed the effects of miR-663a on cell apoptosis under ER stress (Fig. 4G and I and Fig. S4H). Taken together, these results suggest that miR-663a promoted ER stress-

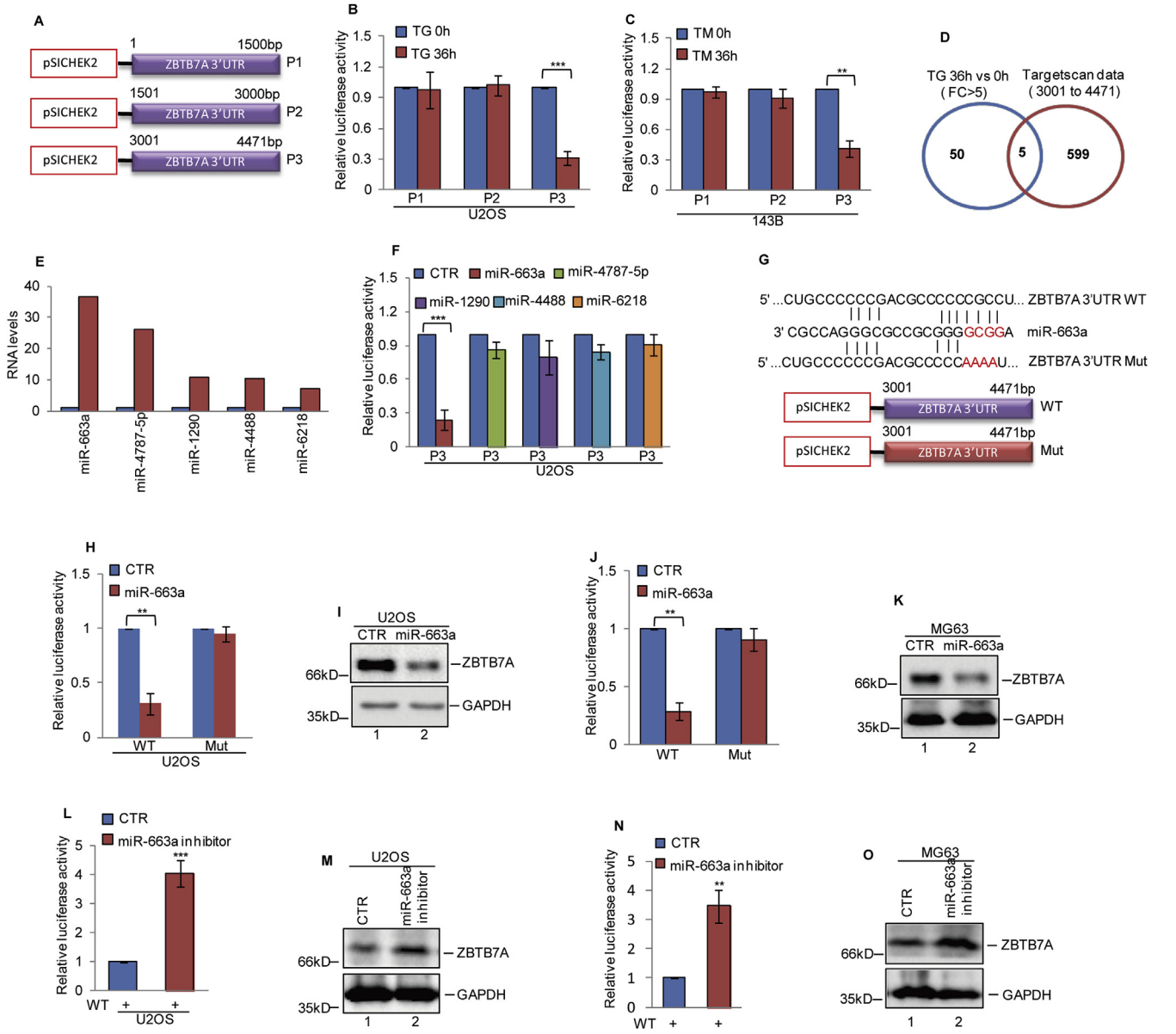


Fig. 3. miR-663a inhibited ZBTB7A expression in osteosarcoma cells. (A–C) Sequences of ZBTB7A 3′UTR were constructed into a pSICHECK2 vector and they were then transfected into U2OS cells. After 1 μM TG or 3 μM TM treatment, the luciferase activities of ZBTB7A 3′UTR were measured. Data represent the mean ± SD values of three independent experiments. **p < 0.01 and ***p < 0.001 vs. control. (D–E) U2OS cells were treated with 1 μM TG at the indicated times, and the cells were subjected to miRNA sequencing analysis. The upregulated miRNAs (Fold change > 5) and the predicted miRNAs targeting ZBTB7A from the Targetscan database are listed. (F) The five miRNAs were respectively transfected into U2OS cells together with ZBTB7A 3′UTR. The luciferase activities of ZBTB7A 3′UTR were measured. Data represent the mean ± SD values of three independent experiments. ***p < 0.001 vs. control. (G) The potential binding region of miR-663a on ZBTB7A was predicted by TargetScan. Sequences of ZBTB7A 3′UTR containing the wild type miR-663a binding site (WT) or the mutant (Mut) were constructed into a pSICHECK2 vector where the red area denotes the mutated region. (H–K) The wild type or mutant ZBTB7A 3′UTR was transfected into U2OS and MG63 cells with or without miR-663a overexpression. Luciferase activities were measured. Expression levels of ZBTB7A were detected by western blotting. Data represent the mean ± SD values of three independent experiments. **p < 0.01 vs. control. (L–O) ZBTB7A 3′UTR was transfected into U2OS and MG63 cells with or without miR-663a inhibition. Luciferase activities were measured. Expression levels of ZBTB7A were detected by western blotting. Data represent the mean ± SD values of three independent experiments. **p < 0.01 and ***p < 0.001 vs. control. (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

induced apoptosis by targeting ZBTB7A in osteosarcoma cells.

3.5. ZBTB7A suppresses LncRNA GAS5 expression under ER stress

Accumulating evidence indicates that long non-coding RNAs play an important role in tumourigenesis and our previous study has revealed that ZBTB7A promotes osteosarcoma chemoresistance by suppressing long non-coding RNA LINC00473 expression [13,21]. Thus, we examined whether ZBTB7A suppresses ER stress-induced apoptosis by regulating long non-coding RNAs expression. To this end, we first analysed long non-coding RNAs expression via RNA sequencing analysis and found that LncRNA GAS5, an important tumour suppressor, is significantly enhanced in response to ER stress treatment in U2OS cells (Fig. 5A and C). To confirm this finding, expression levels of GAS5 were measured in U2OS and MG63 cells under ER stress. We found that GAS5 expression was augmented with increasing TG or TM treatment in U2OS cells. However, this increase disappeared in MG63 cells, which is crosscurrent with ZBTB7A expression (Fig. 5D and G).

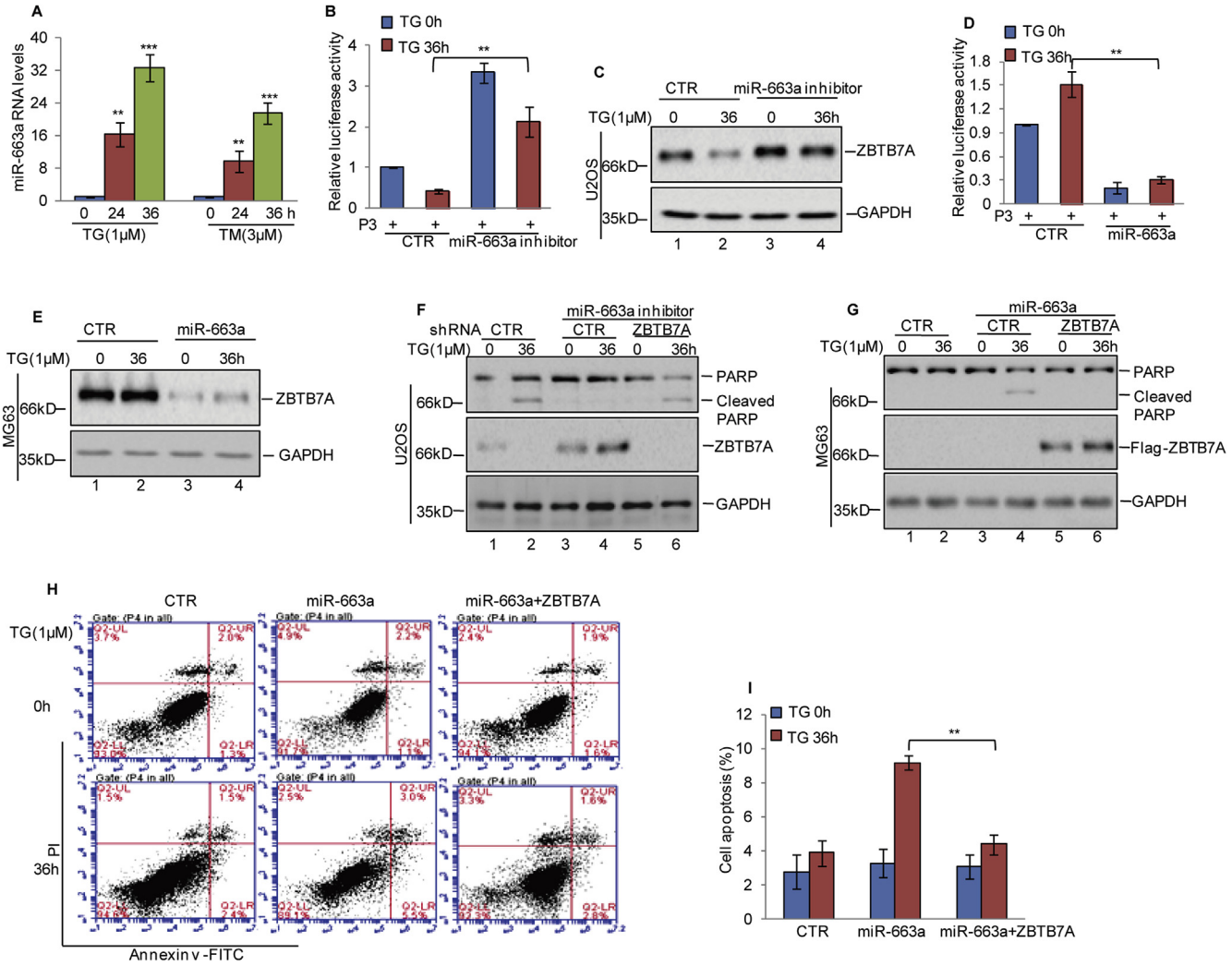


Fig. 4. miR-663a contributed to ER stress-induced ZBTB7A downregulation and promoted cell apoptosis. (A) U2OS cells were treated with 1 μM TG or 3 μM TM at the indicated times and then the expression levels of miR-663a were measured by q-RT-PCR assay. Data represent the mean ± SD values of three independent experiments. **p < 0.01 and ***p < 0.001 vs. control. (B–C) ZBTB7A 3′UTR was transfected into U2OS cells with or without miR-663a inhibitor and then the cells were treated with 1 μM TG for the indicated times. Luciferase activities were measured. Expression levels of ZBTB7A were detected by western blotting. Data represent the mean ± SD values of three independent experiments. **p < 0.01 vs. control. (D–E) ZBTB7A 3′UTR was transfected into MG63 cells with or without miR-663a overexpression and then the cells were treated with 1 μM TG for the indicated times. Luciferase activities were measured. The expression levels of ZBTB7A were detected by western blotting. Data represent the mean ± SD values of three independent experiments. **p < 0.01 vs. control. (F) miR-663a inhibitor was transfected into U2OS cells with or without ZBTB7A knockdown and then the cells were treated with 1 μM TG for the indicated times. Cell apoptosis was detected by western blotting assay. (G–I) miR-663a was transfected into MG63 cells with or without ZBTB7A overexpression and then the cells were treated with 1 μM TG for the indicated times. Cell apoptosis was detected by western blotting and by flow cytometer. Data represent the mean ± SD values of three independent experiments. **p < 0.01 vs. control.

To further prove the relationship between ZBTB7A and GAS5, we instantaneously transfected ZBTB7A or the control vector into U2OS cells and then treated the cells with 1 μM TG or 3 μM TM. Compared to the control cells, we found that ZBTB7A overexpression suppressed ER stress-induced GAS5 expression (Fig. 5H and I and Figs. S5A–5B). Conversely, ZBTB7A knockdown promoted ER stress-induced GAS5 expression in MG63 (Fig. 5J and K and Figs. S5C–5D). Collectively, these data suggest that ZBTB7A is an essential suppressor of ER stress-induced GAS5 expression.

3.6. ZBTB7A binds to the promoter of GAS5 in response to ER stress treatment

To identify potential ZBTB7A-binding regions on the GAS5 promoter, we first cloned the upstream sequence of GAS5 and different truncations by PCR. We then inserted them into pGL3-based luciferase reporter plasmids, which were labelled P1-P2 (Fig. 6A). We subsequently transfected them into U2OS cells with or without 1 μM TG or 3 μM TM treatment. As is shown in Fig. 6B and C, the luciferase activities of P1 but not those of P2 were enhanced in U2OS cells under ER stress treatment, indicating that the region (−1000 to −500 bp) was a key region for GAS5 expression under ER stress.

To further confirm the effects of ZBTB7A on GAS5 promoter, P1 was transfected into U2OS cells with or without ZBTB7A overexpression, and then, the cells were treated with 1 μM TG or 3 μM TM at the indicated times. Fig. 6D and E shows that elevated ZBTB7A eliminated the increase in luciferase activity of P1 under ER stress. In contrast, the inhibition of ZBTB7A elevated the luciferase activity of P1 in response to TG or TM treatment in MG63 cells (Fig. 6F and G). Subsequently performed chromatin immunoprecipitation (ChIP) assays show that chromatin fragments corresponding to the putative ZBTB7A-binding region were specifically present in anti-ZBTB7A immunoprecipitates

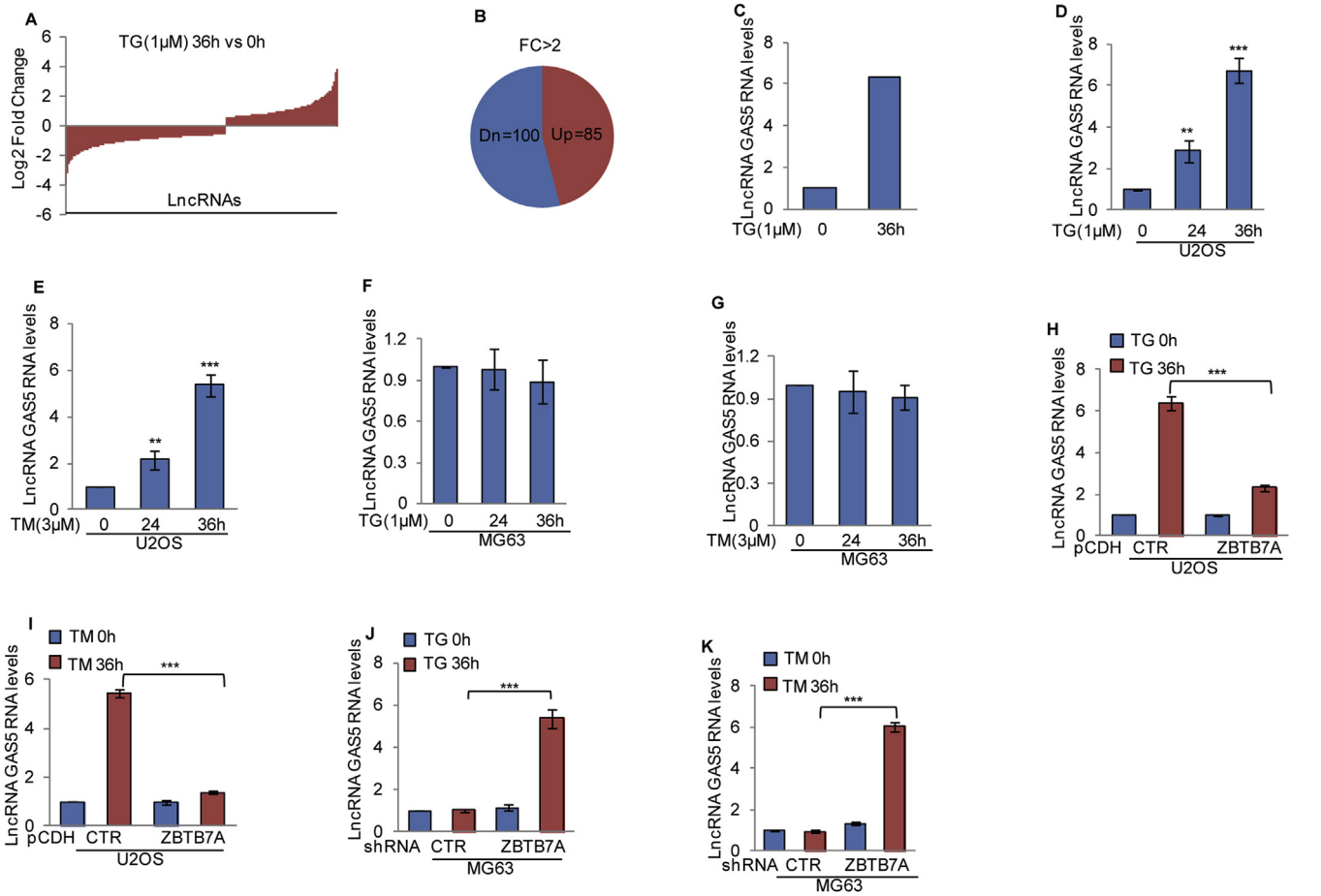


Fig. 5. ZBTB7A repressed LncRNA GAS5 expression under ER stress. (A–C) U2OS cells were treated with 1 μM TG for the indicated times and then the cells were subjected to RNA sequencing analysis. The number of altered LncRNAs was determined. (D–G) U2OS and MG63 cells were treated with 1 μM TG or 3 μM TM for the indicated times. The expression levels of GAS5 were measured by q-RT-PCR. Data represent the mean ± SD values of three independent experiments. **p < 0.01 and ***p < 0.001 vs. control. (H–I) U2OS cells with or without ZBTB7A overexpression were treated with 1 μM TG or 3 μM TM for the indicated times. The expression levels of GAS5 were measured by q-RT-PCR. Data represent the mean ± SD values of three independent experiments. **p < 0.01 and ***p < 0.001 vs. control. (J–K) MG63 cells with or without ZBTB7A knockdown were treated with 1 μM TG or 3 μM TM for the indicated times. Expression levels of GAS5 were measured by q-RT-PCR. Data represent the mean ± SD values of three independent experiments. **p < 0.01 and ***p < 0.001 vs. control.

from MG63 cells in response to ER stress treatment (Fig. 6H and I). Taken together, these data indicate that ZBTB7A can bind to the promoter of GAS5, leading to its decline in ER stress resistant cells with TG or TM treatment.

3.7. The miR-663a-ZBTB7A-GAS5 axis plays an important role in osteosarcoma adaptation to ER stress

To determine the functional significance of the miR-663a-ZBTB7A-GAS5 pathway to osteosarcoma adaptation to ER stress, we first determined the effect of GAS5 on ER stress-induced apoptosis in osteosarcoma cells and found that the knockdown of GAS5 inhibits ER stress-induced apoptosis in U2OS cells (Fig. 7A and B and Figs. S6A–4B). Conversely, the overexpression of GAS5 in MG63 cells promoted cell apoptosis (Fig. 7C and D and Figs. S6C–4D). We transfected GAS5 into ZBTB7A overexpressing U2OS cells and found that the effects of ZBTB7A on cell apoptosis and cell viability were reversed by GAS5 overexpression (Fig. 7E and F). Similarly, GAS5 knockdown eliminated increases in ZBTB7A depletion-induced cell apoptosis and decreases in cell viability under ER stress (Fig. 7G and H). These data indicate that ZBTB7A enhanced osteosarcoma adaptation to ER stress by suppressing GAS5 expression.

Considering our data showing that miR-663a inhibits ZBTB7A expression and that the latter functions in ER stress-induced apoptosis in osteosarcoma cells, we hypothesized that miR-663a serves pro-apoptotic functions by upregulating GAS5 expression. To prove this finding, we first transfected miR-663a inhibitor into U2OS cells and treated the cells with 1 μM TG. Compared to the control cells, we found that inhibition of miR-663a eliminated ER stress-induced GAS5 upregulation (Fig. 7I). Consistently, the elevation of miR-663a in MG63 cells promoted GAS5 expression (Fig. 7J). We then overexpressed GAS5 in U2OS cells with or without miR-663a inhibition and the cells were treated with 1 μM TG. We found that the increase in GAS5 recovered cell apoptosis, which was inhibited by miR-663a inhibitor under ER stress (Fig. 7K and L). A similar result was obtained for MG63 cells (Fig. 7M and N).

4. Discussion

In this work we provide evidence showing that ZBTB7A plays a key role in the survival of osteosarcoma cells undergoing pharmacological ER stress-induced by TM or TG. ZBTB7A was downregulated upon TM or TG treatment in osteosarcoma cells that were relatively sensitive to ER stress, and the exogenous expression of ZBTB7A inhibited ER stress-induced apoptosis in these cells. Conversely, the knockdown of ZBTB7A in ER stress resistant osteosarcoma cells rendered the cells susceptible to elimination by TM or TG. Moreover, our further mechanistic studies revealed that the upregulation of miR-663a directly bound to the 3′UTR

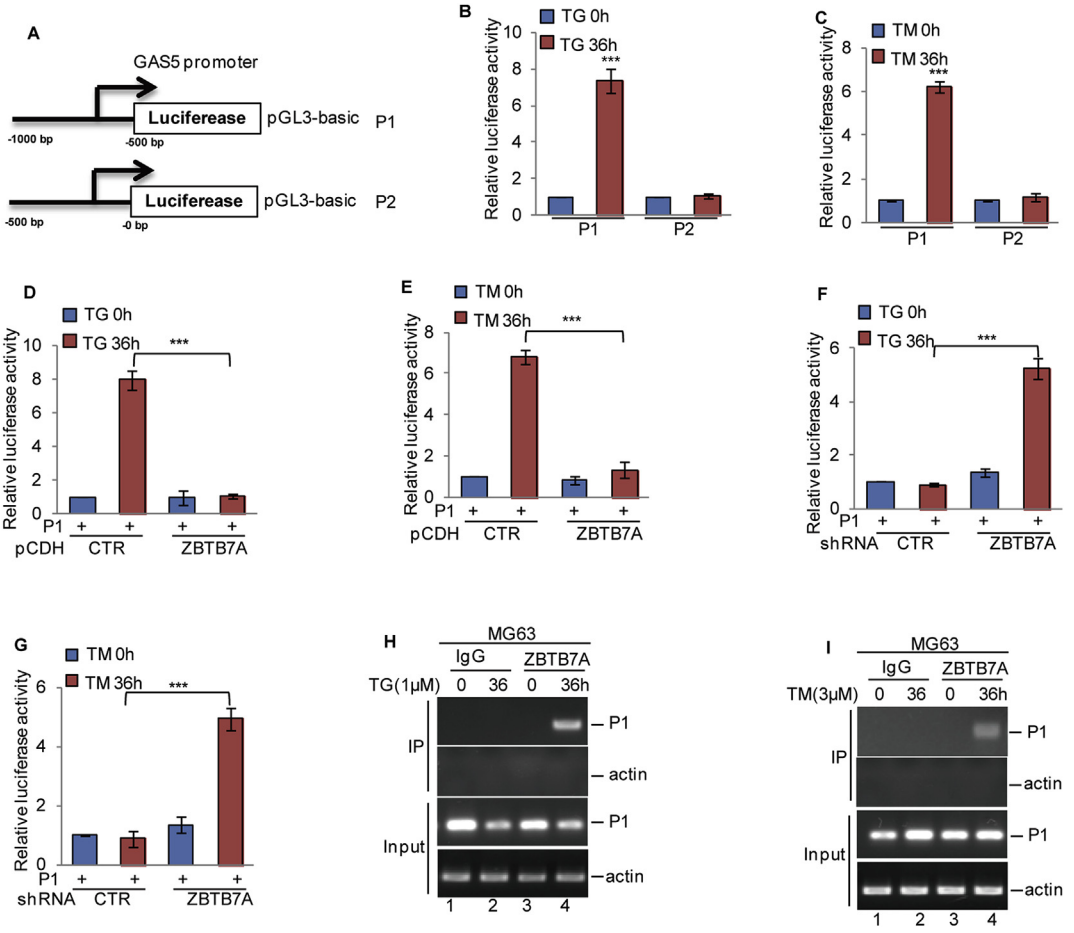
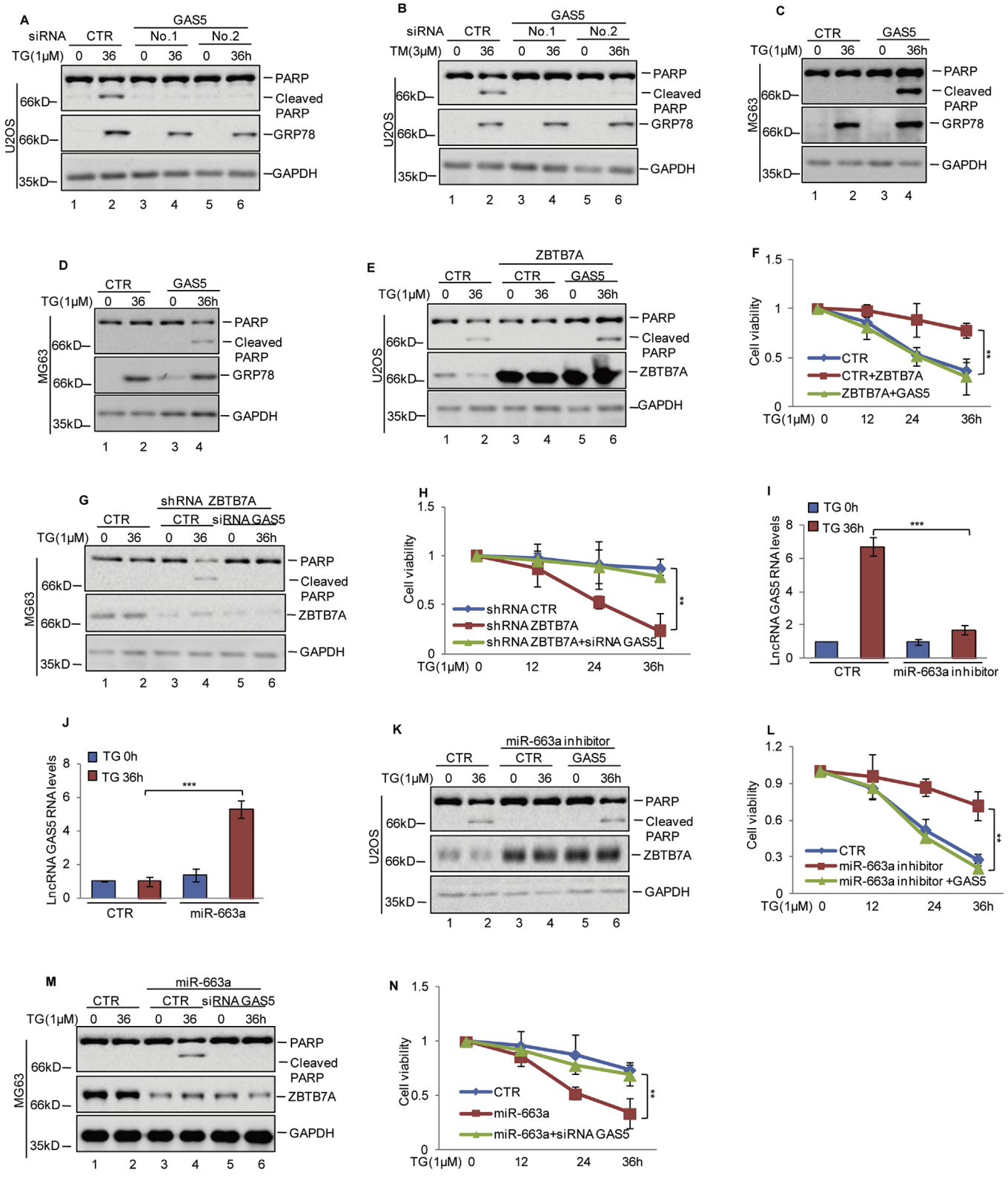


Fig. 6. ZBTB7A bound to the promoter of GAS5. (A) Schematic illustration of pGL3-based reported constructs were used in luciferase assays to examine the transcriptional activity of GAS5. (B–C) Parts of the promoter of GAS5, named P1 and P2, were individually transfected into U2OS cells with or without 1 μM TG or 3 μM TM treatment. Luciferase activity was measured. Data represent the mean ± SD values of three independent experiments. ***p < 0.001 vs. control. (D–E) The promoter of GAS5, named P1, was transfected into U2OS cells with or without ZBTB7A overexpression and then the cells were treated with 1 μM TG or 3 μM TM for the indicated times. Luciferase activity was measured. Data represent the mean ± SD values of three independent experiments. ***p < 0.001 vs. control. (F–G) The promoter of GAS5, named P1, was transfected into MG63 cells with or without ZBTB7A knockdown and then the cells were treated with 1 μM TG or 3 μM TM for the indicated times. Luciferase activity was measured. Data represent the mean ± SD values of three independent experiments. ***p < 0.001 vs. control. (H–I) ChIP analysis showing the binding of ZBTB7A to the promoter of GAS5 in MG63 cells under 1 μM TG or 3 μM TM treatment. An isotype-matched IgG was used as a negative control.

of ZBTB7A and contributed to ZBTB7A downregulation under ER stress in U2OS and 143B cells. Our results also show that the inhibition of ER stress-induced apoptosis and increased metastasis by ZBTB7A in osteosarcoma cells occurs due to the transcriptional suppression of GAS5 expression(Figs. S7A–7C). Thus, our data reveal the prosurvival role of ZBTB7A in osteosarcoma adaptation to ER stress.

Accumulating evidence shows that ZBTB7A can medicate both cell survival and death signalling in different types of tumours. The frequent loss of ZBTB7A has been found in many forms of human carcinoma and the overexpression of ZBTB7A has been reported to suppress cell growth and metastasis in lung cancer and melanoma [11,22–24]. In particular, the tumour suppressive role of ZBTB7A was implicated in a study showing the loss of ZBTB7A augmented tumourigenesis of mouse prostate cancer in a Pten-deficient background [25]. However, ZBTB7A is originally known as a proto-oncoprotein due to its ability to suppress the transcription of tumour suppressor gene ARF [9]. The overexpression of ZBTB7A in immature mice of T and B lymphoid lineage leads to aggressive lymphomas consistent with the proto-oncogenic role of ZBTB7A in lymphoma [26]. In osteosarcoma, our previous data suggested that ZBTB7A was increased in cisplatin-resistant cells and inhibited cisplatin-induced apoptosis. In this study, we found that ZBTB7A is important for the survival of osteosarcoma cells undergoing pharmacological ER stress and that downregulation of ZBTB7A in osteosarcoma accelerated cell apoptosis under ER stress treatment. In normal condition, the effect of ZBTB7A on cell apoptosis was disappeared indicating that the role of ZBTB7A in osteosarcoma cell apoptosis was relied on the ER stress. After that, we also found that ZBTB7A was elevated in osteosarcoma tissues and associated with the tumour metastasis.

Previous studies indicated that some microRNAs involved in regulating ZBTB7A expression such as miR-100 suppressed tumour growth and metastasis by targeting ZBTB7A. Similarly, our data also showed that miR-663a induced by ER stress directly bound to the 3′UTR of ZBTB7A and led to ZBTB7A downregulation in ER stress sensitive osteosarcoma cells under TG or TM treatment. Previous studies have indicated that miR-663a plays roles in many crucial biological functions such as those of cell proliferation, differentiation, and apoptosis in several types of tumours. miR-663a is highly expressed and promotes cancer progression in lung, ovarian, breast, nasopharyngeal and gallbladder cancer [27–30]. However, miR-663a has also been reported to exhibit tumour suppressor properties in glioblastoma, pancreatic cancer, and gastric cancer [31–33]. A recent study showed that ER stress-induced upregulates miR-663a and directly inhibits PLOD3 expression [34]. Similarly, our data showed that miR-663a directly



(caption on next page)

suppressed ZBTB7A expression and facilitated ER stress-induced apoptosis in osteosarcoma cells. However, the molecular mechanism of miR-663a upregulation in response to ER stress should be studied in the future.

How does ZBTB7A promote osteosarcoma cell survival under pharmacological ER stress? Our data showed that ZBTB7A bound to the promoter of GAS5 in response to ER stress treatment and protected cells from ER stress-induced apoptosis. GAS5 is a transcript of the growth arrest-specific 5 (GAS5) gene that was first isolated in 1988 in search of novel tumour suppressors by the subtractive cDNA cloning of genes which are preferentially expressed in growth-arrested cells. Many studies have indicated that GAS5 is dysregulated in multiple cancers and is

Fig. 7. miR-663a-ZBTB7A-lncRNA GAS5 axis regulated osteosarcoma adaptation to ER stress. (A–B) U2OS cells with or without GAS5 knockdown were treated with 1 μM TG or TM. Cell apoptosis was analysed by western blotting. (C–D) MG63 cells with or without GAS5 overexpression were treated with 1 μM TG or 3 μM TM. Cell apoptosis was analysed by western blotting. (E–F) GAS5 was transfected in U2OS cells with or without ZBTB7A overexpression and the cells were treated with 1 μM TG for the indicated times. Cell apoptosis was analysed by western blotting. Cell viability was measured by CCK8 assay. Data represent the mean ± SD values of three independent experiments. **p < 0.01 vs. control. (G–H) GAS5 was knocked down in MG63 cells with or without ZBTB7A inhibition and the cells were treated with 1 μM TG for the indicated times. Cell apoptosis was analysed by western blotting. Cell viability was measured by CCK8 assay. Data represent the mean ± SD values of three independent experiments. **p < 0.01 vs. control. (I) miR-663a inhibitor was introduced into U2OS cells and then the cells were treated with 1 μM TG for the indicated times. The expression levels of GAS5 were measured by q-RT-PCR. Data represent the mean ± SD values of three independent experiments. ***p < 0.001 vs. control. (J) miR-663a was introduced into MG63 cells and then the cells were treated with 1 μM TG for the indicated times. The expression levels of GAS5 were measured by q-RT-PCR. Data represent the mean ± SD values of three independent experiments. ***p < 0.001 vs. control. (K–L) GAS5 was overexpressed in U2OS cells with or without miR-663a inhibitor and the cells were treated with 1 μM TG for the indicated times. Cell apoptosis was analysed by western blotting. Cell viability was measured by CCK8 assay. Data represent the mean ± SD values of three independent experiments. **p < 0.01 vs. control. (M-N) GAS5 was knocked down in MG63 cells with or without miR-663a overexpression and the cells were treated with 1 μM TG for the indicated times. Cell apoptosis was analysed by western blotting. Cell viability was measured by CCK8 assay. Data represent the mean ± SD values of three independent experiments. **p < 0.01 vs. control.

an important tumour suppressor [35–37]. We consistently showed that GAS5 was enhanced under ER stress and that elevated GAS5 promoted cell apoptosis. Previous reports have shown that GAS5 could act as a competing endogenous RNA for some microRNAs and regulated the downstream pathway of these micrRNAs including miR-221, miR-26a and miR-21 [38–40]. Therefore, we will uncover how GAS5 promoted ER stress-induced apoptosis in the future work.

In summary, our data suggest that ZBTB7A plays a prosurvival role in osteosarcoma adaptation to ER stress and that the miR-663a-ZBTB7A-LncRNAGAS5 pathway is essential to the survival of human osteosarcoma cells under ER stress.

Conflicts of interest

The authors have no competing interests to declare.

Acknowledgements

This work was supported with grants from the National Natural Science Foundation of China (81874136 to Chuanchun Han and 31270999 to Wenzhi Zhao) and with a Liaoning Provincial Natural Science Foundation of China (No. 20170540298) grant given to Lu Zhang.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.canlet.2019.01.046.

References

[1] S.S. Bielack, B. Kempf-Bielack, G. Delling, G.U. Exner, S. Flege, K. Helmke, R. Kotz, M. Salzer-Kuntschik, M. Werner, W. Winkelmann, A. Zoubek, H. Jurgens, K. Winkler, Prognostic factors in high-grade osteosarcoma of the extremities or trunk: an analysis of 1,702 patients treated on neoadjuvant cooperative osteosarcoma study group protocols, J. Clin. Oncol.: Off. J. Am. Soc. Clin. Oncol. 20 (2002) 776–790.

[2] G. Ottaviani, N. Jaffe, The epidemiology of osteosarcoma, Cancer Treat Res. 152 (2009) 3–13.

[3] W. Zhou, M. Hao, X. Du, K. Chen, G. Wang, J. Yang, Advances in targeted therapy for osteosarcoma, Discov. Med. 17 (2014) 301–307.

[4] D.X. Nguyen, P.D. Bos, J. Massague, Metastasis: from dissemination to organ-specific colonization, Nat. Rev. Canc. 9 (2009) 274–284.

[5] D.T. Rutkowski, R.J. Kaufman, A trip to the ER: coping with stress, Trends Cell Biol. 14 (2004) 20–28.

[6] A. Papaioannou, E. Chevet, Driving cancer tumorigenesis and metastasis through UPR signaling, Curr. Top. Microbiol. Immunol. 414 (2018) 159–192.

[7] C.J. Liu, L. Prazak, M. Fajardo, S. Yu, N. Tyagi, P.E. Di Cesare, Leukemia/lymphoma-related factor, a POZ domain-containing transcriptional repressor, interacts with histone deacetylase-1 and inhibits cartilage oligomeric matrix protein gene expression and chondrogenesis, J. Biol. Chem. 279 (2004) 47081–47091.

[8] J.A. Costoya, Functional analysis of the role of POK transcriptional repressors, Briefings Funct. Genomics Proteomics 6 (2007) 8–18.

[9] T. Maeda, R.M. Hobbs, T. Merghoub, I. Guernah, A. Zelent, C. Cordon-Cardo, J. Teruya-Feldstein, P.P. Pandolfi, Role of the proto-oncogene Pokemon in cellular transformation and ARF repression, Nature 433 (2005) 278–285.

[10] D.B. Shi, Y.W. Wang, A.Y. Xing, J.W. Gao, H. Zhang, X.Y. Guo, P. Gao, C/EBPalpha-induced miR-100 expression suppresses tumor metastasis and growth by targeting ZBTB7A in gastric cancer, Cancer Lett. 369 (2015) 376–385.

[11] Y. Liu, Y. Li, J. Liu, Y. Wu, Q. Zhu, MicroRNA-132 inhibits cell growth and metastasis in osteosarcoma cell lines possibly by targeting Sox4, Int. J. Oncol. 47 (2015) 1672–1684.

[12] X.S. Liu, J.E. Haines, E.K. Mehanna, M.D. Genet, I. Ben-Sahra, J.M. Asara, B.D. Manning, Z.M. Yuan, ZBTB7A acts as a tumor suppressor through the transcriptional repression of glycolysis, Genes Dev. 28 (2014) 1917–1928.

[13] L. Zhang, Y. Wang, X. Li, X. Xia, N. Li, R. He, H. He, C. Han, W. Zhao, ZBTB7A enhances osteosarcoma chemoresistance by transcriptionally repressing lncRNALINC00473-IL24 activity, Neoplasia 19 (2017) 908–918.

[14] R. Jiang, C. Zhang, G. Liu, R. Gu, H. Wu, MicroRNA-101 inhibits proliferation, migration and invasion in osteosarcoma cells by targeting ROCK1, American journal of cancer research 7 (2017) 88–97.

[15] Y. Mei, Z. Du, C. Hu, N.F. Greenwald, M. Abedalthagafi, N.Y.R. Agar, G.P. Dunn, W.L. Bi, S. Santagata, I.F. Dunn, Osteoglycin promotes meningioma development through downregulation of NF2 and activation of mTOR signaling, Cell Commun. Signal.: CCS 15 (2017) 34.

[16] X. Li, C. Ma, L. Zhang, N. Li, X. Zhang, J. He, R. He, M. Shao, J. Wang, L. Kang, C. Han, LncRNAAC132217.4, a KLF8-regulated long non-coding RNA, facilitates oral squamous cell carcinoma metastasis by upregulating IGF2 expression, Cancer Lett. 407 (2017) 45–56.

[17] B. Ma, Z. Yuan, L. Zhang, P. Lv, T. Yang, J. Gao, N. Pan, Q. Wu, J. Lou, C. Han, B. Zhang, Long non-coding RNA AC023115.3 suppresses chemoresistance of glioblastoma by reducing autophagy, Biochim. Biophys. Acta 1864 (2017) 1393–1404.

[18] X. Yang, T. Du, X. Wang, Y. Zhang, W. Hu, X. Du, L. Miao, C. Han, IDH1, a CHOP and C/EBPbeta-responsive gene under ER stress, sensitizes human melanoma cells to hypoxia-induced apoptosis, Cancer Lett. 365 (2015) 201–210.

[19] N. Hojo, N. Tatsumi, N. Moriguchi, A. Matsumura, S. Morimoto, J. Nakata, F. Fujiki, S. Nishida, H. Nakajima, A. Tsuboi, Y. Oka, N. Hosen, S. Hayashi, H. Sugiyama, Y. Oji, A Zbtb7a proto-oncogene as a novel target for miR-125a, Mol. Carcinog. 55 (2016) 2001–2009.

[20] X. Liang, Q. Zhao, T. Geng, S. Luo, Q. He, MiR-106b regulates the apoptosis and tumorigenesis of hepatocellular carcinoma via targeting Zinc finger and BTB domain-containing protein 7A (Zbtb7a), J. Biochem. Mol. Toxicol. 32 (2018) e22169.

[21] A. Sanchez Calle, Y. Kawamura, Y. Yamamoto, F. Takeshita, T. Ochiya, Emerging roles of long non-coding RNA in cancer, Cancer Sci. 109 (2018) 2093–2100.

[22] R. Beroukhim, C.H. Mermel, D. Porter, G. Wei, S. Raychaudhuri, J. Donovan, J. Barretina, J.S. Boehm, J. Dobson, M. Urashima, K.T. Mc Henry, R.M. Pinchback, A.H. Ligon, Y.J. Cho, L. Haery, H. Greulich, M. Reich, W. Winckler, M.S. Lawrence, B.A. Weir, K.E. Tanaka, D.Y. Chiang, A.J. Bass, A. Loo, C. Hoffman, J. Prensner, T. Liefeld, Q. Gao, D. Yecies, S. Signoretti, E. Maher, F.J. Kaye, H. Sasaki, J.E. Tepper, J.A. Fletcher, J. Tabernero, J. Baselga, M.S. Tsao, F. Demichelis, M.A. Rubin, P.A. Janne, M.J. Daly, C. Nucera, R.L. Levine, B.L. Ebert, S. Gabriel, A.K. Rustgi, C.R. Antonescu, M. Ladanyi, A. Letai, L.A. Garraway, M. Loda, D.G. Beer, L.D. True, A. Okamoto, S.L. Pomeroy, S. Singer, T.R. Golub, E.S. Lander, G. Getz, W.R. Sellers, M. Meyerson, The landscape of somatic copy-number alteration across human cancers, Nature 463 (2010) 899–905.

[23] T.I. Zack, S.E. Schumacher, S.L. Carter, A.D. Cherniack, G. Saksena, B. Tabak, M.S. Lawrence, C.Z. Zhsng, J. Wala, C.H. Mermel, C. Sougnez, S.B. Gabriel, B. Hernandez, H. Shen, P.W. Laird, G. Getz, M. Meyerson, R. Beroukhim, Pan-cancer patterns of somatic copy number alteration, Nat. Genet. 45 (2013) 1134–1140.

[24] H. Alam, N. Li, S.S. Dhar, S.J. Wu, J. Lv, K. Chen, E.R. Flores, L. Baseler, M.G. Lee, HP1gamma promotes lung Adenocarcinoma by downregulating the transcription-repressive regulators NCOR2 and ZBTB7A, Cancer Res. 78 (2018) 3834–3848.

[25] G. Wang, A. Lunardi, J. Zhang, Z. Chen, U. Ala, K.A. Webster, Y. Tay, E. Gonzalez-Billalabeitia, A. Egia, D.R. Shaffer, B. Carver, X.S. Liu, R. Taulli, W.P. Kuo, C. Nardella, S. Signoretti, C. Cordon-Cardo, W.L. Gerald, P.P. Pandolfi, Zbtb7a suppresses prostate cancer through repression of a Sox9-dependent pathway for cellular senescence bypass and tumor invasion, Nat. Genet. 45 (2013) 739–746.

[26] T. Maeda, T. Merghoub, R.M. Hobbs, L. Dong, M. Maeda, J. Zakrzewski, M.R. van den Brink, A. Zelent, H. Shigematsu, K. Akashi, J. Teruya-Feldstein, G. Cattoretti, P.P. Pandolfi, Regulation of B versus T lymphoid lineage fate decision by the proto-oncogene LRF, Science 316 (2007) 860–866.

[27] Z.Y. Liu, G.L. Zhang, M.M. Wang, Y.N. Xiong, H.Q. Cui, MicroRNA-663 targets

TGFB1 and regulates lung cancer proliferation, Asian Pac. J. Cancer Prev. APJCP: Asian Pac. J. Cancer Prev. APJCP 12 (2011) 2819–2823.
[28] H. Hu, S. Li, X. Cui, X. Lv, Y. Jiao, F. Yu, H. Yao, E. Song, Y. Chen, M. Wang, L. Lin, The overexpression of hypomethylated miR-663 induces chemotherapy resistance in human breast cancer cells by targeting heparin sulfate proteoglycan 2 (HSPG2), J. Biol. Chem. 288 (2013) 10973–10985.
[29] C. Yi, Q. Wang, L. Wang, Y. Huang, L. Li, L. Liu, X. Zhou, G. Xie, T. Kang, H. Wang, M. Zeng, J. Ma, Y. Zeng, J.P. Yun, MiR-663, a microRNA targeting p21(WAF1/CIP1), promotes the proliferation and tumorigenesis of nasopharyngeal carcinoma, Oncogene 31 (2012) 4421–4433.
[30] Q. Ma, Y. Zhang, H. Liang, F. Zhang, F. Liu, S. Chen, Y. Hu, L. Jiang, Y. Hao, M. Li, Y. Liu, EMP3, which is regulated by miR-663a, suppresses gallbladder cancer progression via interference with the MAPK/ERK pathway, Cancer Lett. 430 (2018) 97–108.
[31] J. Pan, H. Hu, Z. Zhou, L. Sun, L. Peng, L. Yu, L. Sun, J. Liu, Z. Yang, Y. Ran, Tumor-suppressive mir-663 gene induces mitotic catastrophe growth arrest in human gastric cancer cells, Oncol. Rep. 24 (2010) 105–112.
[32] Y. Shi, C. Chen, S.Z. Yu, Q. Liu, J. Rao, H.R. Zhang, H.L. Xiao, T.W. Fu, H. Long, Z.C. He, K. Zhou, X.H. Yao, Y.H. Cui, X. Zhang, Y.F. Ping, X.W. Bian, miR-663 suppresses oncogenic function of CXCR4 in glioblastoma, Clin. Cancer Res.: Off. J. Am. Assoc. Canc. Res. 21 (2015) 4004–4013.
[33] W. Zang, Y. Wang, T. Wang, Y. Du, X. Chen, M. Li, G. Zhao, miR-663 attenuates tumor growth and invasiveness by targeting eEF1A2 in pancreatic cancer, Mol. Canc. 14 (2015) 37.
[34] G. Amodio, E. Sasso, C. D'Ambrosio, A. Scaloni, O. Moltedo, S. Franceschelli, N. Zambrano, P. Remondelli, Identification of a microRNA (miR-663a) induced by ER stress and its target gene PLOD3 by a combined microRNome and proteome approach, Cell Biol. Toxicol. 32 (2016) 285–303.
[35] C. Ma, X. Shi, Q. Zhu, Q. Li, Y. Liu, Y. Yao, Y. Song, The growth arrest-specific transcript 5 (GAS5): a pivotal tumor suppressor long noncoding RNA in human cancers, Tumour Biol.: J. Inter. Soc. Oncodevelopmental Biol. Med. 37 (2016) 1437–1444.
[36] M.R. Pickard, G.T. Williams, Molecular and cellular mechanisms of action of tumour suppressor GAS5 LncRNA, Genes 6 (2015) 484–499.
[37] W. Sun, Y. Yang, C. Xu, J. Guo, Regulatory mechanisms of long noncoding RNAs on gene expression in cancers, Cancer genetics 216–217 (2017) 105–110.
[38] K. Ye, S. Wang, H. Zhang, H. Han, B. Ma, W. Nan, Long noncoding RNA GAS5 suppresses cell growth and epithelial-mesenchymal transition in osteosarcoma by regulating the mir-221/ARHI pathway, J. Cell. Biochem. 118 (2017) 4772–4781.
[39] W. Liang, T. Fan, L. Liu, L. Zhang, Knockdown of growth-arrest specific transcript 5 restores oxidized low-density lipoprotein-induced impaired autophagy flux via upregulating miR-26a in human endothelial cells, Eur. J. Pharmacol. 843 (2019) 154–161.
[40] Z. Zhang, Z. Zhu, K. Watabe, X. Zhang, C. Bai, M. Xu, F. Wu, Y.Y. Mo, Negative regulation of lncRNA GAS5 by miR-21, Cell Death Differ. 20 (2013) 1558–1568.