

Original Article

ZFX-mediated down-regulation of FBP1 confers to growth in melanoma

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Abstract: Melanoma is one of the most aggressive and lethal cancers. The mechanisms for melanoma progression are still not fully elucidated. Here, we demonstrated that FBP1 (Fructose-1,6-bisphosphatase) expression was frequently down-regulated in melanoma tissues. FBP1 down-regulation inversely associated with prognosis in melanoma patients. Restored FBP1 expression inhibited glucose uptake and lactate production in melanoma cells. Restored FBP1 expression also inhibited melanoma cells proliferation under hypoxia in vitro, and inhibited melanoma growth in vivo. Additionally, ZFX (zinc finger protein, X-linked) was identified to bind to the *FBP1* promoter to transcriptionally inhibit FBP1 expression in melanoma cells, and conversely correlated with FBP1 expression in melanoma. These findings support that FBP1 down-regulation confers to the progression of melanoma. Identification of the ZFX-FBP1 signaling axis as a biomarker of prognosis in melanoma will be valuable in future development of therapeutic strategies aimed at improving treatment efficacy.

Keywords: Melanoma, FBP1, tumor growth, ZFX

Introduction

Melanoma is one of the most aggressive and lethal cancers. Approximately 76,100 new melanoma cases with 9,710 deaths are estimated in the United States only in 2014 [1]. Despite recent advances in therapeutic strategies, melanoma with distant metastasis still portends a poor prognosis with a 5-year survival rate of 16%, because most patients are diagnosed at the time with metastatic disease [2]. However, the molecular mechanisms underlying the development and progression of melanoma have been poorly understood so far.

Glucose homeostasis is reciprocally controlled by the catabolic glycolysis/oxidative phosphorylation (OXPHOS) and the anabolic gluconeogenesis pathway. In the catabolic reaction, the process of glycolysis, glucose is converted to pyruvate and then metabolized to lactate under the hypoxic condition. Under the normoxic condition, pyruvate is channeled to the TCA (tricarboxylic acid) cycle to fuel OXPHOS in the mitochondria. Several decades ago, Otto Warburg has reported for the first time that tumor cells

consume glucose at a significantly higher rate than normal cells, and that tumor cells preferentially metabolized glucose to lactate even in the presence of ample oxygen, a process termed aerobic glycolysis [3, 4]. Several molecular events and signaling pathways have been reported to contribute to the Warburg effect in tumor cells. For example, tumor cells can increase an embryonic form of pyruvate kinase M2 (PKM2) to trigger glycolysis in lung cancer [5]. MACC1 enhances the Warburg effect to supports human gastric cancer growth under metabolic stress [6]. RRAD, a small GTPase, inhibits the Warburg effect through negative regulation of the NF- κ B signaling [7]. So far, most reports have focused on the regulation of the catabolic pathway of glucose, but gluconeogenesis is less investigated which may also play important role in the switch to aerobic glycolysis in tumor cells. Fructose-1,6-bisphosphatase (FBP) is an important gluconeogenesis regulatory enzyme. FBP catalyzes the hydrolysis of fructose-1,6-bisphosphate to fructose-6-phosphate and inorganic phosphate. The fructose-1,6-bisphosphate is one of the most important intermediate in glycolysis and its level is mainly

controlled by fructose-6-phosphate kinase and FBP. There are two isoenzymes of FBP in mammalian cells, FBP1 and FBP2. The FBP1 is ubiquitously expressed in different tissues while FBP2 expression is restricted to muscle tissues. Loss of FBP1 expression increases glucose uptake and glycolysis, and then leads to hypoglycemia and lactic acidosis. FBP1 was originally identified as a rate-limiting enzyme in gluconeogenesis. Recently, FBP1 acts as a tumor suppressor has been found to be down-regulated in several human cancer types, such as gastric cancer [8], hepatocellular carcinoma and colon cancer [9], and basal-like breast cancer [10]. FBP1 down-regulation has been associated with the Warburg effect which is a common metabolic characteristic of cancer cells. However, the expression pattern and role of FBP1, and mechanisms underlying FBP1 down-regulation in melanoma remains unclear. Here, we demonstrated the frequent down-regulation of FBP1 expression in melanoma, which was correlated with a poor prognosis. Restored FBP1 expression inhibited melanoma cells proliferation and melanoma growth. Additionally, we identified ZFX as a negative regulator of FBP1 expression via direct binding to *FBP1* promoter in melanoma cells.

Materials and methods

Clinical specimens and cell culture

7 paired fresh melanoma tissues and adjacent morphologically normal skin tissues, and 67 formalin-fixed paraffin-embedded tissues were obtained at Renmin Hospital of Wuhan University (Hubei, China). Fresh tissue samples were immediately snap-frozen in liquid nitrogen used for mRNA and protein extraction. The study was approved by each of the patients and by the ethics committee of Renmin Hospital of Wuhan University. The human melanoma cell lines A375 and MeWo were obtained from the American Type Culture Collection (ATCC, Manassas, VA, USA). Cells were cultured in RPMI-160 medium supplemented with 10% fetal bovine serum (Gibco, Carlsbad, CA, USA). The HEK293T human embryonic kidney cell line was cultured in DMEM (Gibco) with 10% fetal bovine serum.

Real-time PCR (qRT-PCR) for mRNA

Total RNA was extracted with a Trizol protocol, and cDNAs from the mRNAs were synthesized

with the Super-Script first-strand synthesis system (Thermo Scientific, Glen Brunie, MA, USA). Real-time PCR was carried out according to the standard protocol on ABI 7500 with SYBR Green detection (Applied Biosystems, Foster City, CA, USA). GAPDH was used as an internal control and the qRT-PCR was repeated three times. The primers for GAPDH were: forward primer 5'-ATTCCATGGCACCGTCAAGGCTGA-3', reverse primer 5'-TTCTCCATGGTGGTGAAGACGCCA-3'; for FBP1 were: forward primer 5'-CGCGCACCTCTATGGCATT-3', reverse primer 5'-TTCTTCTGACACGAGAACACAC-3'. The gene expression threshold cycle (CT) value was calculated by normalizing with internal control GAPDH and relative quantization values were calculated.

Western blot

Total proteins were extracted from corresponding cells using the RIPA buffer in the presence of Protease Inhibitor Cocktail (Thermo Scientific, Rockford, IL, USA). The protein concentration of the lysates was measured using a BCA Protein Assay Kit (Thermo Scientific). Equivalent amounts of protein were resolved and mixed with 5× Lane Marker Reducing Sample Buffer, electrophoresed in a 10% SDS-acrylamide gel and transferred onto Immobilon-P Transfer Membrane (Merck Millipore, Schwalbach, Germany). The membranes were blocked with 5% non-fat milk in Tris-buffered saline and then incubated with primary antibodies followed by secondary antibody. The signal was detected using an ECL detection system (Merck Millipore). The FBP1 antibody was from Santa Cruz Biotechnology (Dallas, Texas, USA). The ZFX antibody, TXNIP antibody and β -Actin antibody were from Cell Signaling Technology. HRP-conjugated secondary antibody was from Thermo.

Cells transfection

Cells were trypsinised, counted and seeded onto 6-well plates the day before transfection to ensure 70% cell confluence on the day of transfection. The transfection of pLEX-FBP1 vector and control vector was carried out using Lipofectamine 2000 (Invitrogen, Carlsbad, CA, USA) in accordance with the manufacturer's procedure. Puromycin was used to select stable clone. The siRNAs target to ZFX and control were purchased from Santa Cruz Biotechnology. The transfection of 50 nM siRNA or control was

performed as above. At 48 h post-transfection, follow-up experiments were performed.

Metabolic assays

Glucose uptake and lactate production were measured by assay kits from BioVision (San Francisco, CA, USA). Briefly, cells were seeded at a density of 2000 cells per well in a 96 well plate. Cells were washed with PBS and starved overnight in 100 μ l serum free medium. Cells were washed three times with PBS, and starved for glucose by preincubated with 100 μ l KRPH buffer containing 2% BSA for 40 min. 10 μ l of 10 mM 2-DG was added and incubated for 20 min. For each well, 50 μ l Reaction Mix was added and incubated at 37°C for 40 min, protected from light. Then fluorescence was measured at Ex/Em = 535/587 nm and the glucose uptake rate was calculated. For lactate production, 25 μ l culture medium from cells were added to a 96-well plate. Adjust the volume to 50 μ l per well with Lactate Assay Buffer. 50 μ l Reaction Mix was added to each well and incubated at room temperature for 30 min, protected from light. Then the absorbance (OD 570 nm) was measured on the BioTek Synergy 2 and the lactate production rate was calculated.

Cell proliferation assay

Cell proliferation was monitored by the MTS assay using the CellTiter96@AQueous One Solution Cell Proliferation Assaykit (Promega, Madison, WI, USA) according to the manufacturer's instructions. Related treated cells were seeded into 96-well plates at 2000 cells/well (200 μ l/well). The cell proliferation assay was performed on days 2 by incubation with MTS (20 μ l/well). After 2 h further incubation, the absorbance at 490 nm was recorded for each well on the BioTek Synergy 2. The absorbance represented the cell number and the absorbance from control was set as 100%.

Xenograft model in nude mice

Xenograft tumors were generated by subcutaneous injection of FBP1 overexpressing or control melanoma cancer cells (2×10^6), into the hind limbs of 4-6 week-old Balb/C athymic nude mice. All mice were housed and maintained under specific pathogen-free conditions, and all experiments were approved by the Use

Committee for Animal Care and performed in accordance with institutional guidelines. Tumor formation was examined every 3 days for the whole duration of the experiment. Tumors were harvested and weighed at the experimental endpoint.

ChIP assay

The ChIP assay was performed using EZ-CHIPTM chromatin immunoprecipitation kit (Merck Millipore). In brief: Chromatin proteins were crosslinked to DNA by addition of formaldehyde to the culture medium to a final concentration of 1%. After incubation for 10 min at room temperature, the cells were washed and scraped off in ice-cold PBS containing Protease Inhibitor Cocktail II. Cells were pelleted and then resuspended in Lysis Buffer containing Protease Inhibitor Cocktail II. The resulting lysate was subjected to sonication to reduce the size of DNA to 200-1000 bp. The sample was centrifuged to remove cell debris and diluted 10-fold in ChIP dilution buffer containing Protease Inhibitor Cocktail II. Remove 5 μ l of the supernatant as "Input" and save at 4°C. Then 5 μ g of IgG (as negative control) or anti-ZFX antibody (cell signal technology) were added to the chromatin solution and incubated overnight at 4°C with rotation. After antibody incubation, the protein G agarose was added and incubated at 4°C with rotation for 2 h. The protein/DNA complexes were washed with wash buffers four times and eluted with ChIP Elution Buffer, and cross-links were reversed to free DNA by addition of 5 M NaCl and incubation at 65°C for 4 h. The DNA was purified according to manufacturer's instructions and 50 μ l of DNA from each treatment was obtained. 0.2 μ l of DNA from each group was used as plate for PCR. The primers for FBP promoter with ZFX binding sites were as followed, sense: 5'-CCGCAGGGAGGATCCCCGACC-3', antisense: 5'-GCGCTCGTGTGCAACTGGGCC-3'. The PCR samples were resolved by electrophoresis in a 2% agarose gel and stained with ethidium bromide.

Immunohistochemistry

The sections were dried at 55°C for 2 h and then deparaffinized in xylene and rehydrated using a series of graded alcohol washes. The tissue slides were then treated with 3% hydrogen peroxide in methanol for 15 min to quench endogenous peroxidase activity and antigen

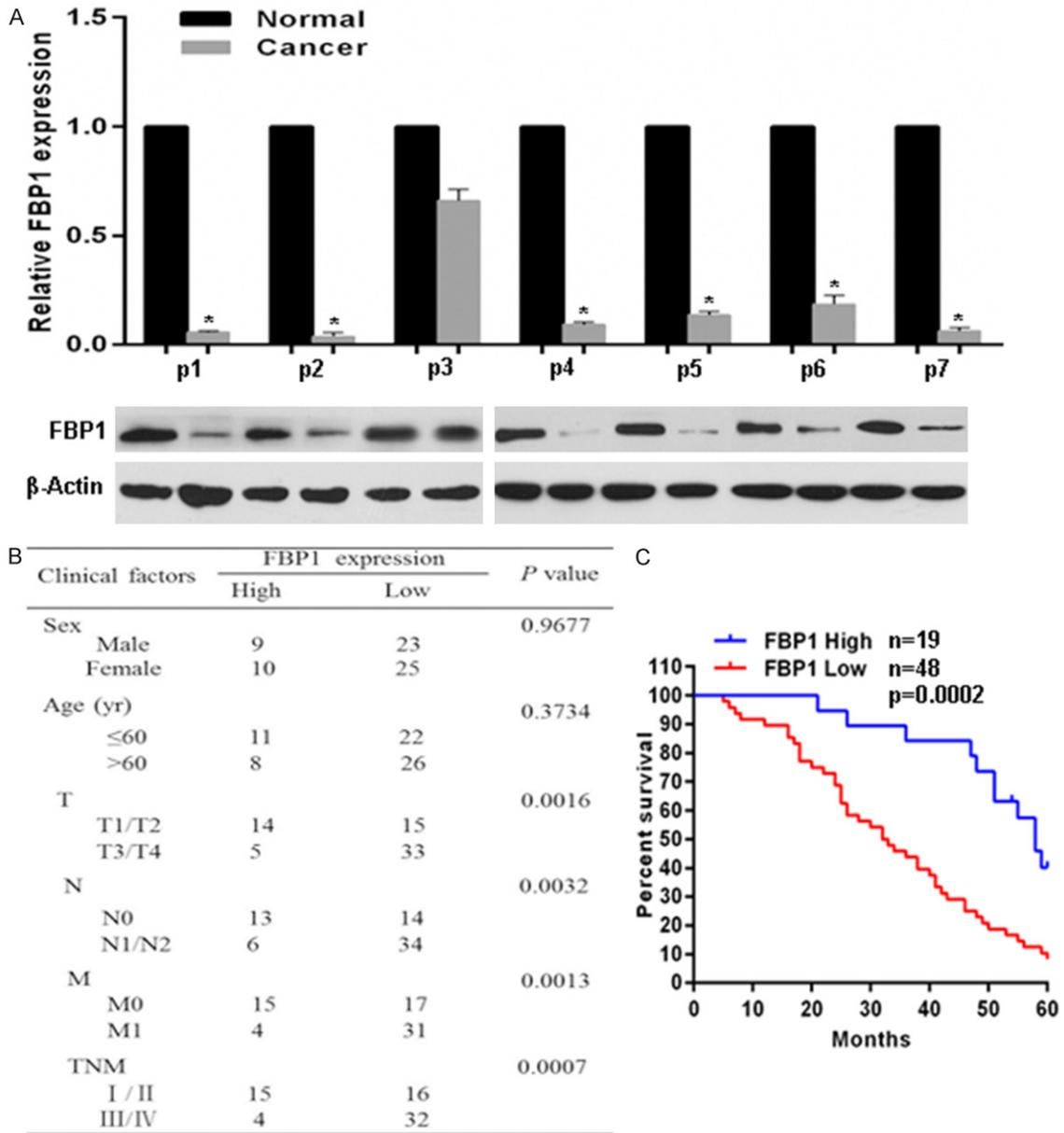


Figure 1. FBP1 expression is down-regulated in melanoma. A. The expression pattern of FBP1 in human melanoma and their adjacent non-malignant tissues was detected by qRT-PCR and western blot. vs. normal, *P < 0.01. B. The correlation analysis of FBP1 protein expression in relation to clinicopathologic variables of melanoma patients. C. Kaplan-Meier analysis estimated the overall survival according to FBP1 protein level.

retrieval then performed by incubation in 0.01 M sodium citrate buffer (pH 6.0) and heating using a microwave oven. After a 1 h preincubation in 10% goat serum, the specimens were incubated with primary antibody overnight at 4°C. The tissue slides were treated with a non-biotin horseradish peroxidase detection system according to the manufacturer's instruction (DAKO, Glostrup, Denmark). Two different pathologists evaluated the immunohistological samples.

Statistical analysis

All statistical analyses were performed with SPSS statistical software (version 21.0; IBM). Survival curves were constructed using the Kaplan-Meier method and analyzed by the log-rank test. Significant prognostic factors identified by univariate analysis were entered into multivariate analysis using the Cox proportional hazards model. The Student's t-test was used for comparisons and the Pearson correlation

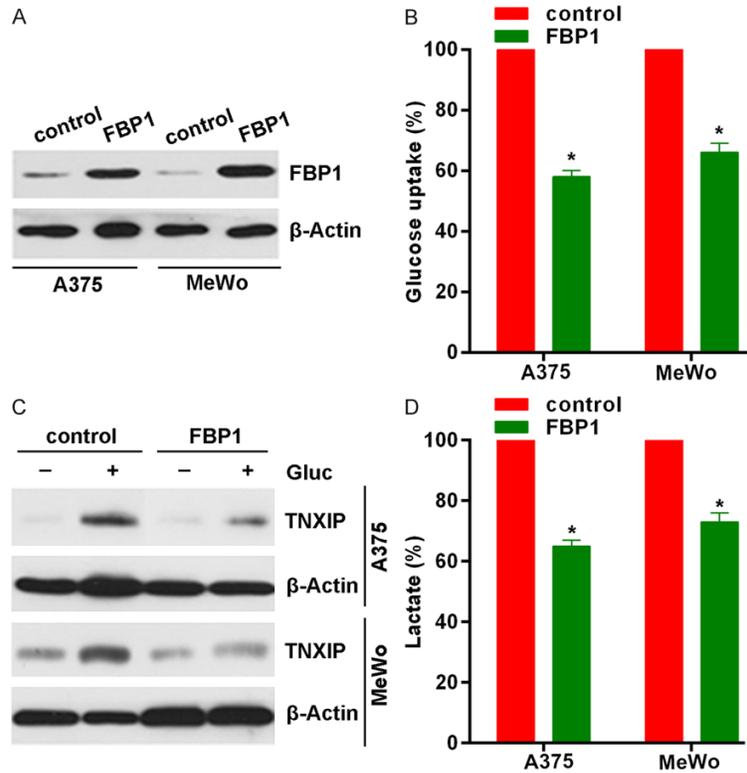


Figure 2. FBP1 inhibits glucose uptake and sensitivity in melanoma cells. A. The stable FBP1-overexpressed melanoma cell lines were established with transfection of FBP1 expressing plasmid pLEX-FBP1. B. Glucose uptake was measured after FBP1 overexpression. vs. control, * $P < 0.01$. C. Cells were deprived for glucose for 12 h followed by glucose stimulation for additional 3 h. TXNIP expression was examined by western blot to determine the glucose sensitivity. D. Lactate excretion was measured after FBP1 overexpression. vs. control, * $P < 0.01$.

test (two-tailed) was used to investigate the correlation between ZFX and FBP1 protein levels. Statistical significance was defined as $P < 0.05$.

Results

FBP1 down-regulation associated with advanced disease in melanoma

To investigate the expression pattern of FBP1 in melanoma, we firstly detected FBP1 expression with qRT-PCR and western blot in freshly collected 7 pairs of human melanoma and adjacent non-tumor tissues. As shown, FBP1 mRNA and protein levels were significantly down-regulated in melanoma tissues (**Figure 1A**). Additionally, we also detected FBP1 protein level with IHC in formalin-fixed paraffin-embedded tissues from 67 cases of melanoma tissues. Results showed that FBP1 protein level

was markedly down-regulated in 48 out of 67 melanoma tissues (**Figure 4E**). FBP1 down-regulation is correlated with disease progression of human melanoma (**Figure 1B**). Importantly, melanoma patients with low FBP1 expression have a poor prognosis for overall survival (**Figure 1C**). These results imply that FBP1 down-regulation correlates with melanoma progression and is a negative prognostic marker.

FBP1 inhibits glucose uptake and sensitivity in melanoma cells

Because FBP1 expression is down-regulated in melanoma tissues, we wonder the biological role of FBP1 in melanoma cells. We established stable FBP1 overexpression in melanoma A375 and MeWo cell lines (**Figure 2A**). Then, we measured glucose uptake and found that FBP1 overexpression significantly decreased glucose uptake in A375 and MeWo cell lines (**Figure 2B**). MondoA and ChREBP are

the sensors of intracellular glucose level. Following an increase in intracellular glucose-derived metabolites, MondoA and ChREBP shuttle to the nucleus, and then interact with Mix and activate transcription of target genes. Among the targets, TXNIP is a major direct target of MondoA-Mlx complex and is commonly used as an intracellular glucose sensor [10, 11]. So, here we examined TXNIP induction by depleting glucose for 12 hours, followed by glucose stimulation for 3 hours. We found that TXNIP was markedly induced in A375 and MeWo cell lines, but FBP1 overexpression suppressed TXNIP induction (**Figure 2C**). To examine whether FBP1 inhibits aerobic glycolysis, we detected lactate production and found that FBP1 overexpression reduced lactate production in A375 and MeWo cell lines (**Figure 2D**). These results suggest that FBP1 is involved in glucose metabolism and inhibits glycolysis in melanoma cells.

ZFX-FBP1 axis in melanoma

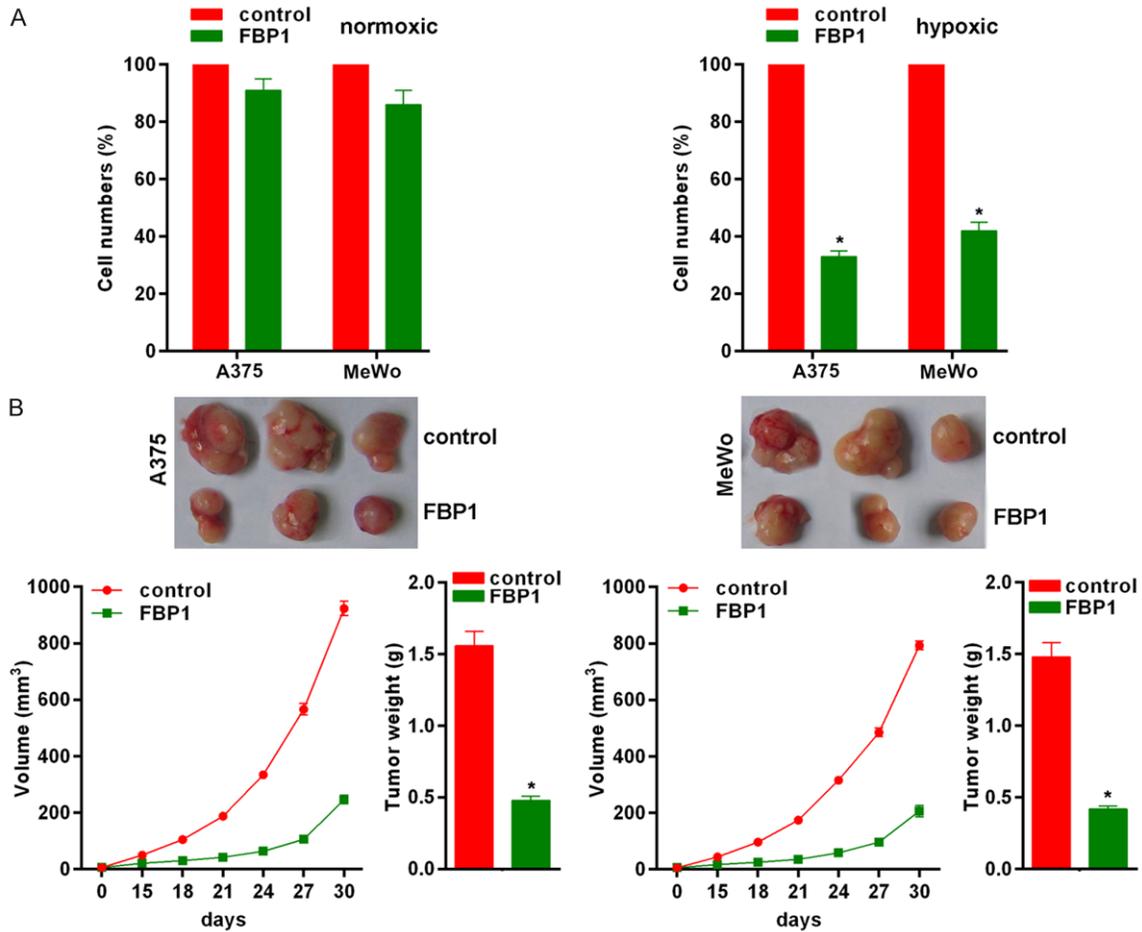


Figure 3. FBP1 inhibits melanoma cells growth. A. Cell growth was measured by MTS assay for 2 days culture. Data are presented as a percentage of control values. B. Tumor growth was monitored every 3 days. Tumor size and weight were recorded. vs. control, *P < 0.01.

FBP1 inhibits melanoma cells growth

To verify the functions of FBP1 in regulating melanoma cell proliferation, we firstly quantitatively analyzed the effect of FBP1 overexpression on cell proliferation in melanoma A375 and MeWo cell lines by MTS assay. Results demonstrated FBP1 overexpression induced a drastic proliferation inhibition under hypoxic condition (0.1% oxygen), but with a minor effect under normoxic condition (21% oxygen) (**Figure 3A**). To confirm the roles of FBP1 in the regulation of melanoma growth, we analyzed the effect of FBP1 on tumorigenicity in vivo using a xenograft mouse model. FBP1-overexpressed A375 and MeWo cell lines and their related control cell lines were injected subcutaneously into the flank of nude mice. We found FBP1 overexpress significantly suppressed melanoma growth derived from A375 and MeWo cell

lines (**Figure 3B**). These results revealed that restored FBP1 expression inhibits melanoma cells growth in vitro and in vivo.

ZFX transcriptionally inhibits FBP1 expression in melanoma cells

We have shown above that FBP1 mRNA and protein levels were significantly down-regulated in melanoma tissues. To investigate whether transcriptional regulation contributes to FBP1 down-regulation in melanoma cells, we analyzed the response elements of a cohort of transcription factors located within a two kb region upstream of the initiation codon (ATG) of FBP1 gene using JASPAR database (<http://jaspar.binf.ku.dk>) and we noticed that there are two putative ZFX binding sites within this region (**Figure 4A**). To confirm the direct association of ZFX with the *FBP1* promoter, we performed

ZFX-FBP1 axis in melanoma

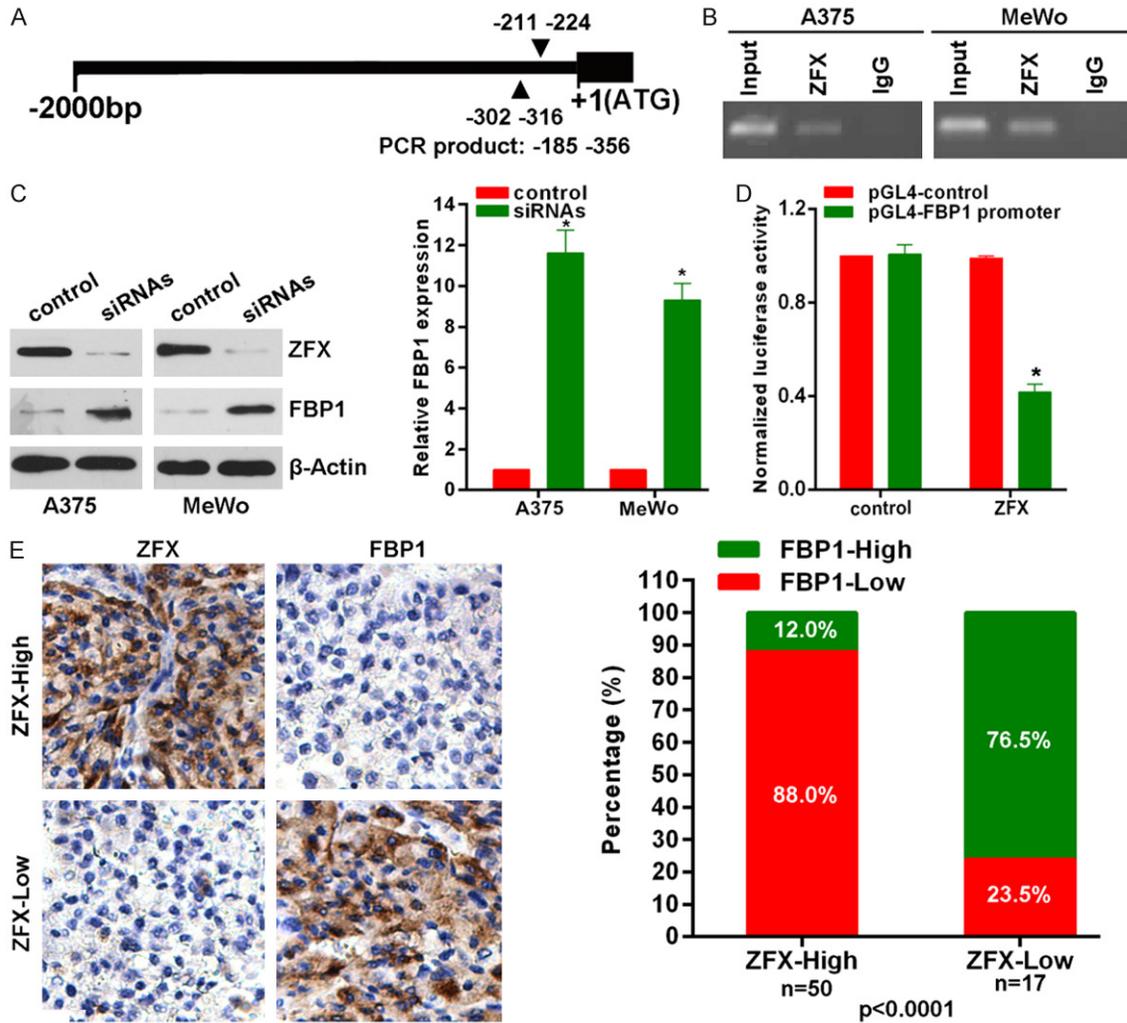


Figure 4. ZFX inhibits FBP1 expression in melanoma cells. A. A schematic representation of ZFX binding site in the 2 kb putative *FBP1* promoter upstream of the initiation codon (ATG). B. ChIP assays showed ZFX physically bound to the *FBP1* promoter in melanoma cells. C. The *FBP1* expression after ZFX knockdown was determined by qRT-PCR and western blot. vs. control, **P* < 0.01. D. ZFX inhibited the luciferase activity driven by *FBP1* promoter in HEK293T cells. vs. related control, **P* < 0.01. E. Representative images of ZFX and *FBP1* protein expression in melanoma tissues. ZFX protein expression was inversely correlated with that of *FBP1*.

ChIP assays in A375 and MeWo cells for the putative ZFX binding sites within the two kilobase region. ChIP results revealed that ZFX bound exactly to the potential *FBP1* promoter (Figure 4B). Expectedly, ZFX knockdown significantly up-regulated *FBP1* mRNA and protein expression in A375 and MeWo cells (Figure 4C). To determine further the effects of ZFX on *FBP1* expression, the putative two kilobase *FBP1* promoter was cloned into a luciferase reporter vector and expression assays subsequently performed. As expected, in addition, ZFX was found to significantly suppressed luciferase activity driven by the *FBP1* promoter in HEK293T cells (Figure 4D). Importantly, *FBP1*

protein expression decreased in 44 of 50 melanoma tissues that exhibited high ZFX protein level (Figure 4E). In contrast, *FBP1* protein expression decreased only in 4 of 17 melanoma tissues that exhibited low ZFX protein level (Figure 4E). These results indicate that ZFX can directly bind to the *FBP1* promoter to transcriptionally down-regulate *FBP1* expression in melanoma cells.

Discussion

In this study, we demonstrated that *FBP1* is down-regulated in melanoma and its down-regulation is inversely associated with prognosis in

melanoma patients. Restored FBP1 expression inhibited glycolysis in melanoma cells and inhibited melanoma cells proliferation in vitro and in vivo. FBP1 deficiency is correlated with an autosomal recessive inherited disorder which is characterized with hypoglycemia and lactic acidosis [12]. Loss of FBP1 maybe increase glucose uptake and glycolysis, then led to hypoglycemia and lactic acidosis. In type 2 diabetic mouse models, loss of FBP1 also increases glucose sensitivity and utilization [13]. FBP1 as tumor suppressor has been reported in several studies. Liu et al. indicated the down-regulation of FBP1 in gastric cancer and overexpressed FBP1 suppressed proliferation and glycolysis in gastric cancer cells [8]. In hepatocellular carcinoma and colon cancer, FBP1 was also reported to be down-regulated and restored FBP1 expression induced G2-M phase cell cycle arrest to inhibit cell growth [9]. Recently, Dong et al. reported loss of FBP1 by snail1 mediated the metabolic reprogramming resulted into an increased cancer stem cell-like property and tumorigenicity in basal-like breast cancer [10]. In addition to the role in metabolic reprogramming, FBP1 can restrain renal carcinoma progression via inhibiting nuclear hypoxia-inducible factors (HIFs) function [14]. Although the reported potential role of FBP1 in human cancer, the exact role and mechanisms in cancer development and progression still need further studies.

FBP1 expression is down-regulated in reported human cancer types. The mechanism for down-regulation of FBP1 is suggested to be associated with DNA methylation in FBP1 promoter. As the downstream of Ras, NF- κ B promotes DNA methylation to down-regulated FBP1 expression in gastric cancer [8]. In basal-like breast cancer cells, snail-G9a-Dnmt1 complex mediated the promoter DNA methylation for the loss expression of FBP1 [10]. To determine whether the epigenetic silencing is responsible for the down-regulation of FBP1 expression in melanoma, we found 5-aza-2'-dC treatment did not significantly changed FBP1 mRNA and protein levels in melanoma cells (data not shown). To identify transcription factors conferring to down-regulation of FBP1 in melanoma cells, we found ZFX physically binds to the FBP1 promoter and inhibits the activity of FBP1 promoter. ZFX knockdown significantly enhanced FBP1 expression in mRNA and protein levels in mel-

noma cells. ZFX protein level was inversely correlated with FBP1 protein level in melanoma tissues. ZFX (zinc finger protein, X-linked) is a transcription factor that is highly conserved in vertebrates and contains a large acidic transcriptional activation domain and a C-terminal zinc finger domain [15]. ZFX plays a key role in controlling the self-renew and maintenance of both embryonic stem cells and hematopoietic stem cells [16, 17]. ZFX is also found to be frequently up-regulated in a number of human cancer types involved in regulation of cell proliferation, cell cycle distribution, apoptosis, cell survival and cancer stemness, such as esophageal carcinoma [18], gastric cancer [19], prostate cancer [20], lung cancer [21], glioma [22], hepatocellular carcinoma [23], gallbladder cancer [24] and skin basal cell carcinoma [25]. Here, although ZFX was identified to transcriptionally regulate FBP1 expression in melanoma cells, the exact biological role and detailed mechanisms remain to be explored in our further studies.

In summary, we here demonstrated the down-regulation of FBP1 expression in melanoma and restored FBP1 expression inhibited melanoma cell growth, accompanied with metabolic switch from glycolysis to OXPHOS. ZFX expression inversely correlated with FBP1 expression in melanoma and transcriptionally inhibited FBP1 expression. Our findings strongly suggest that the ZFX-FBP1 axis can be a prognostic biomarker for melanoma patients and provides a rationale for the development of anticancer intervention strategies targeting the ZFX-FBP1 axis in clinics.

Disclosure of conflict of interest

None.

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