FBW7 (F-box and WD Repeat Domain-Containing 7) Negatively Regulates Glucose Metabolism by Targeting the c-Myc/TXNIP (Thioredoxin-Binding Protein) Axis in Pancreatic Cancer

Shunrong Ji1,2,3, Yi Qin1,2,3, Chen Liang1,2,3, Run Huang4, Si Shi1,2,3, Jiang Liu1,2,3, Kaizhou Jin1,2,3, Dingkong Liang1,2,3, Wenyan Xu1,2,3, Bo Zhang1,2,3, Liang Liu1,2,3, Chen Liu1,2,3, Jin Xu1,2,3, Quanxing Ni1,2,3, Paul J. Chiao5, Min Li6, and Xianjun Yu1,2,3

Abstract

Purpose: FBW7 functions as a tumor suppressor by targeting oncoproteins for destruction. We previously reported that the oncogenic mutation of KRAS inhibits the tumor suppressor FBW7 via the Ras–Raf–MEK–ERK pathway, which facilitates the proliferation and survival of pancreatic cancer cells. However, the underlying mechanism by which FBW7 suppresses pancreatic cancer remains unexplored. Here, we sought to elucidate the function of FBW7 in pancreatic cancer glucose metabolism and malignancy.

Experimental Design: Combining maximum standardized uptake value (SUVmax), which was obtained preoperatively via a PET/CT scan, with immunohistochemistry staining, we analyzed the correlation between SUVmax and FBW7 expression in pancreatic cancer tissues. The impact of FBW7 on glucose metabolism was further validated in vitro and in vivo. Finally, gene expression profiling was performed to identify core signaling pathways.

Results: The expression level of FBW7 was negatively associated with SUVmax in pancreatic cancer patients. FBW7 significantly suppressed glucose metabolism in pancreatic cancer cells in vitro. Using a xenograft model, MicroPET/CT imaging results indicated that FBW7 substantially decreased 18F-fluorodeoxyglucose (18F-FDG) uptake in xenograft tumors. Gene expression profiling data revealed that TXNIP, a negative regulator of metabolic transformation, was a downstream target of FBW7. Mechanistically, we demonstrated that TXNIP was a c-Myc target gene and that FBW7 regulated TXNIP expression in a c-Myc-dependent manner.

Conclusions: Our results thus reveal that FBW7 serves as a negative regulator of glucose metabolism through regulation of the c-Myc/TXNIP axis in pancreatic cancer. Clin Cancer Res; 22(15); 3950–60. ©2016 AACR.

Introduction

Pancreatic cancer is a devastating disease and is the fourth leading cause of cancer-related deaths in the United States (1). Pancreatic ductal adenocarcinoma (PDAC) accounts for approximately 95% of pancreatic cancer cases (2). Due to late diagnosis, high metastatic potential, and resistance to chemoradiotherapy, there are no effective treatments for refractory pancreatic cancer (3–5). Hence, there is an urgent need for an increased understanding of the biologic characteristics and molecular mechanisms of pancreatic cancer.

F-box and WD repeat domain-containing 7 (FBW7) is the substrate recognition component for the Skp1-Cul1-F-box (SCF) ubiquitin ligase complex and targets many oncoproteins for destruction. Loss of the tumor-suppressive function of FBW7 has been proposed to drive the progression of multiple cancers. Deletion or mutation of FBW7 has been frequently identified in many cancers, including gastric cancer, colon cancer, and breast carcinoma (6). Overall, approximately 6% of human tumors harbor FBW7 mutations. Emerging evidence has shown that FBW7 is also regulated by multiple upstream genes, such as p53, Pin1, Hex-5, and Numb4, as well as by miRNAs (7). We previously reported that fewer than 2% of pancreatic cancer samples harbored FBW7 mutations, according to sequencing analysis (8). Furthermore, with mass spectrometry analysis, we detected that ERK kinase phosphorylated FBW7 at the T205 site, which resulted in destabilization of FBW7 in pancreatic cancer. However, the exact role of FBW7 in pancreatic cancer progression has not been investigated.

Pancreatic cancer is characterized by extensive desmoplasia that is caused by the dense stromal fibroinflammatory reaction of fibroblasts, which leads to a reduced nutrient and oxygen supply, resulting in a severe hypoxic tumor microenvironment (9, 10). To adapt and survive in this hostile environment, cancer cells must
FBW7 Inhibits Glucose Metabolism in Pancreatic Cancer

Translational Relevance
To assess the potential use of FBW7 in pancreatic cancer diagnosis and prognosis, we combined molecular imaging technology (PET/CT) and immunohistochemistry to evaluate the correlation between SUVmax and FBW7 expression levels. Our clinical and mechanistic findings indicate that FBW7 regulates glucose metabolism through the effector TXNIP, which predicted the poor prognosis of pancreatic cancer by negatively regulating proliferation and glucose metabolism. Moreover, FBW7 regulated TXNIP expression through the E3 ubiquitin ligase substrate c-Myc. Overall, the dysregulation of the FBW7/c-Myc/TXNIP pathway is a promising new target for novel therapeutic inhibitors to treat pancreatic cancer. Furthermore, key signature enzymes of the glycolysis cascade, such as GLUT1, GLUT4, HK2, and LDHA, are also candidate targets for combination treatment regimens. Therefore, our findings may have a critical impact on pancreatic cancer management and may apply to other aggressive and heterogeneous cancers.

Materials and Methods

Cells and reagents
The human pancreatic cancer cell lines with KRAS mutations, SW1990 and PANC-1, were obtained from the American Type Culture Collection. SW1990 cells were cultured in L-15 medium supplemented with 10% FBS. PANC-1 cells were cultured in DMEM supplemented with 10% FBS. All of the cell culture media contained 100 U/mL penicillin and 100 mg/mL streptomycin. The cell lines were authenticated by DNA fingerprinting in 2015 and passaged in our laboratory fewer than 6 months after their receipt. Hypoxia mimetic conditions were chemically generated by treating cells with 200 mmol/L cobalt chloride (CoCl2; Sigma) for the indicated times.

Tissue specimens
The clinical tissue samples used in this study were obtained from patients diagnosed with pancreatic cancer at Fudan University Shanghai Cancer Center from 2010 to 2011. Prior patient consent and approval from the Institutional Research Ethics Committee were obtained. Clinical information regarding the samples is presented in Supplementary Table S1. The pathologic grading was performed by two independent pathologists at our center. The correlation between FBW7 and TXNIP was analyzed using the χ2 test.

Whole-body 18F-FDG PET/CT
Whole-body FDG PET/CT was performed as previously described (24). Briefly, 18F-FDG was automatically made by a cyclotron (Siemens CT I RDS Eclipse ST) using an Explora FDG4 module. Patients had been fasting for more than 6 hours. Scanning started 1 hour after intravenous injection of the tracer (7.4 MBq/kg). The images were acquired on a Siemens biograph 16HR PET/CT scanner with a transaxial intrinsic spatial resolution of 4.1 mm. CT scanning was first initiated from the proximal thighs to the head, with 120 kV, 80–250 mA, pitch 3.6, and rotation time of 0.5 seconds. Image interpretation was carried out on a multimodality computer platform (Syngo; Siemens). Quantification of metabolic activity was acquired using the SUV normalized to body weight, and the SUVmax for each lesion was calculated.

Animal model
BALB/c-nu mice (female, 4 to 6 weeks of age, 18–20 g; Shanghai SLAC Laboratory Animal Co., Ltd.) were housed in sterile filtered cages. The left and right flanks of the mice were injected s.c. with 4 × 106 cells in 100 μL PBS. Six weeks after implantation, the mice were prepared for MicroPET/CT scanning. After scanning, the tumors were surgically dissected. The tumor specimens were fixed in 4% paraformaldehyde. Samples were then processed for histopathologic examination. All animal experiments were performed according to the guidelines for the care and use of laboratory animals and were approved by the Institutional Animal Care and Use Committee of Fudan University.

MicroPET/CT imaging
MicroPET/CT scans and image analyses were performed using an Inveon MicroPET/CT (Siemens Medical Solution). Each tumor-bearing mouse was injected with 11.1 MBq (300 μCi) of...
to the Affymetrix Human HTA2.0 Array for 16 to 18 hours at 45°C. The sample was hybridized after fragmentation of second-cycle single-stranded cDNA (ss-cDNA). A total of 500 ng of RNA was used for a double-round of cDNA synthesis. The sample was purified with magnetic beads from Agencourt Ampure (APN 000132; Beckman Coulter) to generate c-Myc shRNA constructs. The 21-bp target against c-Myc was CCGAGACAGATCCGAAACA.

**Plasmids**

The Flag-tagged coding sequences of human FBW7 and TXNIP were cloned into the lentiviral vector pCDH-CMV-MCS-EF1-puro (SBI) to generate FBW7 expression plasmids. The plKO.1 TRC cloning vector (Addgene plasmid 10878) was used to generate c-Myc shRNA constructs. The 21-bp target against c-Myc was CCGAGACAGATCCGAAACA.

**Cell cycle and cell viability**

Flow cytometric analysis was conducted to examine cell-cycle status using propidium iodide (Invitrogen) and a human Annexin V-FITC kit (Invitrogen), respectively, according to the manufacturer's instructions. All observations were reproduced at least three times in independent experiments.

**Colony-formation assay**

Cells were seeded in triplicate in 6-well plates at an initial density of 500 cells/well. After 10 to 14 days, colonies were visible, and the cells were fixed with 4% paraformaldehyde for 15 minutes at room temperature and stained with 4 mg/mL of crystal violet (Sigma). The colonies containing more than 50 cells were counted using light microscopy. The average number of colonies was determined from three independent experiments.

**Quantitative real-time PCR**

Total RNA was extracted using TRIzol reagent (Invitrogen). cDNA was synthesized by reverse transcription using a TaKaRa PrimeScript RT reagent kit. The expression status of candidate genes and β-actin were determined by quantitative real-time PCR using an ABI 7900HT Real-Time PCR system (Applied Biosystems). All of the reactions were run in triplicate. Primer sequences are listed in Supplementary Table S2.

**RNA extraction, ss-cDNA synthesis, and microarray analysis**

Total RNA from wild-type and FBW7-overexpressing SW1990 cells was extracted with TRIzol/chloroform and then purified with magnetic beads from Agencourt Ampure (APN 000132; Beckman Coulter). Target preparation for microarray processing was carried out according to the GeneChip WT PLUS Reagent Kit. A total of 500 ng of RNA was used for a double-round of cDNA synthesis. After fragmentation of second-cycle single-stranded cDNA (ss-cDNA), the sample was labeled with biotin by terminal deoxynucleotidyl transferase (TdT). Then, the sample was hybridized to the Affymetrix Human HTA2.0 Array for 16 to 18 hours at 45°C. Following the hybridization, the microarrays were washed and stained with streptavidin phycoerythrin on the Affymetrix Fluidics Station 450. The microarrays were scanned by using the Affymetrix GeneChip Command Console (AGCC), which was installed in the GeneChip Scanner3000 7G. The data were analyzed with the Robust Multichip Analysis (RMA) algorithm using the default analysis settings and global scaling as normalization method by Partek Genomics Suite 6.6. Values presented are log_2 RMA signal intensity. The normalized data were further analyzed using one-way ANOVA to screen out the differentially expressed genes. Then, the Database for Annotation, Visualization and Integrated Discovery (DAVID) was used to determine pathways and processes of major biologic significance and importance based on the Gene Ontology annotation function and Kyoto Encyclopedia of Genes and Genomes pathway function. The microarray data were deposited in GEO under accession numbers GSE76443.

**Western blot**

Western blotting was carried out as previously described (8). Briefly, whole-cell protein lysates were extracted. An antibody against FBW7 was purchased from Bethyl. The PGC-1α antibody was purchased from Santa Cruz Biotechnology. The c-Myc and HIF1α antibodies were obtained from Abcam. The TXNIP antibody was produced by ProteinTech. β-Actin was used as a loading control.

**Glycolysis analysis**

Glucose Uptake Colorimetric Assay Kits (Biovision) and Lactate Colorimetric Assay Kits (Biovision) were purchased to examine the glycolysis process in pancreatic cancer cells, according to the manufacturer's protocols.

**Oxygen consumption rate and extracellular acidification rate**

Cellular mitochondrial function was measured using the Seahorse XF Cell Mito stress test Kit and the Bioscience XF96 Extracellular Flux Analyzer, according to the manufacturer's instructions. The glycolytic capacity was determined using the Glycolysis Stress Test Kit as per the manufacturer's instructions. Briefly, 4 × 10^4 cells were seeded onto 96-well plates and incubated overnight. After washing the cells with Seahorse buffer (DMEM with phenol red containing 25 mmol/L glucose, 2 mmol/L sodium pyruvate, and 2 mmol/L glutamine), 175 μL of Seahorse buffer plus 25 μL each of 1 μmol/L oligomycin, 1 μmol/L FCCP, and 1 μmol/L rotenone was automatically injected to measure the oxygen consumption rate (OCR). Then, 25 μL each of 10 mmol/L glucose, 1 μmol/L oligomycin, and 100 mmol/L 2-deoxy-glucose were added to measure the extracellular acidification rate (ECAR). The OCR and ECAR values were calculated after normalization to the cell number and are plotted as the mean ± SD.

**Analysis of ATP production**

The ENLITEN ATP Assay System (Promega; FF2000) was used according to the manufacturer's instructions. Cells were harvested by scraping and were resuspended in PBS. The cell suspension was divided into unequal aliquots. Part of the cell suspension was mixed with 5% trichloroacetic acid (TCA). The remaining cells were used for the cell number calculation. Tris-acetate buffer (pH 7.75) was then added to neutralize the TCA and to dilute the TCA to a final concentration of 0.1%. The diluted sample (40 μL) was added to an equal volume of rL/L reagent (Promega; FF2000). Then, luminescence was measured. The ATP standard (Promega; FF2000) was serially diluted to generate a regression curve for calculating ATP concentrations in individual samples. The relative ATP concentration was determined and normalized to that of the control cells, which was designated as 1. Three independent
experiments were performed. The results are presented as the mean ± SD.

Measurement of mitochondrial membrane potential

The mitochondrial membrane potential assay Kit with JC-1 (Beyotime Biotechnologies) was used to measure the alteration in the mitochondrial membrane potential (ΔΨm). Briefly, cells were harvested by scraping and were resuspended in 0.5 mL of culture medium. Next, 0.5 mL of JC-1 Staining Solution was added to the cell suspension. Then, the suspension was incubated for 20 minutes at 37°C in a CO2 incubator. Next, the cells were collected by centrifugation at 600 × g for 4 minutes. The cells were washed twice with 1 mL of JC-1 Staining Buffer. Subsequently, 500 μL of JC-1 Staining Buffer was added to the cell pellet in each tube, and the cells were thoroughly resuspended. The samples were immediately analyzed using flow cytometry. In healthy cells, JC-1 forms mitochondrial aggregates, which emit red fluorescence at 595 nm when excited at 525 nm. However, after the loss of ΔΨm, JC-1 remains as monomers that emit green fluorescence at 525 nm when excited at 485 nm. Mitochondrial depolarization is indicated by a decrease in the red/green fluorescence intensity ratio.

Chromatin immunoprecipitation assay

Chromatin immunoprecipitation (ChIP) assays were performed using the EZ-ChIP Kit from Millipore according to the manufacturer’s protocol. Primers to detect TXNIP promoter occupancy were: F: 5’-CAGAGCACAACACCAATT-3’ and R: 5’-AGGCTCTGTGCTGCCCTGAC-3’.

siRNA treatments

siRNA duplexes against c-Myc and FBW7 were transfected into pancreatic cancer cells using Lipofectamine 2000 (Invitrogen). The siRNA duplex sense sequences were as follows: si-FBW7-1-5’-ACCTCTCTGAGAGAGAAATGC-3’, si-FBW7-2-5’-GTGTAATGAGACTGGAGA-3’, si-c-Myc-1-5’-CCTGAGACAGTAAGCAA-3’, si-c-Myc-2-5’-CAGTTGAAACACAAACTT-3’.

Statistical analysis

All data are presented as the mean ± SD. Experiments were repeated at least three times. Two-tailed unpaired Student t tests and one-way analysis of variance were used to evaluate the data. SPSS version 16.0 software (IBM) was used for the data analysis. Differences were considered significant at *, P < 0.05; **, P < 0.01; and ***, P < 0.001.

Results

FBW7 expression is negatively correlated with the 18F-FDG PET/CT SUVmax value

18F-FDG PET/CT, which allows for the visualization of the metabolic activity of viable tumor cells, has been widely used in the management of cancer diagnosis. The SUVmax has been widely used as a surrogate marker for the prognosis of numerous types of cancer, including pancreatic cancer (23, 25). To explore the potential relationship between FBW7 and glucose metabolism, we first examined the correlation between the FBW7 IHC staining and the PET/CT SUVmax value. As expected, patients with pancreatic cancer with decreased expression of FBW7 exhibited a higher SUVmax value (Fig. 1A), and the correlation is statistically significant (Fig. 1B). These results indicate that FBW7 plays an inhibitory role in glucose metabolism in pancreatic cancer.

FBW7 inhibits glucose metabolism in pancreatic cancer cells

Glucose metabolism in cancer relies on a series of enzymatic reactions (Fig. 2A). Clinically, in cancer diagnosis, 18F-FDG PET/CT reflects glucose turnover in the tumor lesion. A higher SUVmax value implies an increased glucose metabolic activity in the lesion. To determine the impact of FBW7 expression on cellular metabolism, we constructed PANC-1 and SW1990 stable cell lines ectopically expressing wild-type FBW7 (Fig. 2B). First, we examined glucose uptake and lactate production, two primary indicators of the Warburg effect. As expected, FBW7 decreased glucose uptake and lactate production, indicating its inhibitory role in glycolysis (Fig. 2C and D). The ECAR is another measurement of glucose metabolism and reflects the lactic acid–induced acidification of the medium surrounding cancer cells. FBW7 decreased the ECAR in PANC-1 and SW1990 cells and may play an inhibitory role in lactic acid formed during glycolysis (Fig. 2E).

In addition, cellular oxygen consumption reflects mitochondrial respiration and can be measured by the OCR. PANC-1 and SW1990 cells overexpressing FBW7 exhibited lower OCRs, indicating that FBW7 is a negative regulator of basal mitochondrial respiration (Fig. 2F). Moreover, cancer cells rely on glucose metabolism for ATP production, which meets the demands of rapid proliferation and metastasis. We then analyzed the impact of FBW7 on ATP production. Consistently, FBW7 decreased ATP production in PANC-1 and SW1990 cells (Fig. 2G). Furthermore, the mitochondrial membrane potential, which is used to evaluate early apoptosis, reflects the mitochondrial integrity and varies according to the metabolic state. FBW7 decreased the mitochondrial potential of PANC-1 and SW1990 cells, indicating that FBW7

![Figure 1](https://example.com/figure1.png)

**Figure 1.** Statistical analysis of the correlation between FBW7 expression and the 18F-FDG PET/CT SUVmax. A, representative 18F-FDG PET/CT imaging of PDAC imaging with low or high FBW7 expression (magnification scale bar, 20 μm). B, analysis of the SUVmax in FBW7low and FBW7high groups (n = 60; P < 0.001).
also functions as a negative regulator of mitochondrial glucose metabolism (Fig. 2H). To further explore the role of FBW7 in glucose metabolism, key signature enzymes of the glycolysis cascade were examined. Enzymes related to glucose transport, such as GLUT1, GLUT4, HK2, LDHA, and LDHB, were decreased in FBW7-overexpressing PDAC cells (Fig. 2I). In addition, the levels of other glycolytic enzymes decreased in the presence of FBW7 overexpression (Supplementary Fig. S1A and S1B). Taken together, these results suggest that FBW7 plays a vital role in pancreatic cancer cell glucose metabolism.

FBW7 decreases glucose utilization in a xenograft model
To further confirm the in vitro phenotype of FBW7 in glucose metabolism, we subcutaneously injected nude mice with FBW7-
overexpressing SW1990 cells. As expected, ectopic FBW7 inhibited tumor growth in the xenograft mouse model (Fig. 3A and B). Furthermore, we used a small animal imaging system to evaluate the role of FBW7 in glucose metabolism (Fig. 3C). The results indicated that FBW7 significantly inhibited 18F-FDG uptake in the in vivo xenograft model (Fig. 3D). Subsequent immunohistochemistry using antibodies against GLUT1, GLUT4, HK2, LDHA, and LDHB demonstrated that the expression of these glycolytic enzymes was significantly decreased in tissues from xenograft tumors (Fig. 3E), which was consistent with previous results.

Figure 3.
FBW7 inhibits glucose metabolism in pancreatic cancer. A, SW1990 cells stably expressing FBW7 or empty vector were s.c. injected into nude mice (left, control; right, FBW7). B, at the indicated times, tumors were measured with Vernier calipers (mean ± SEM; n = 5). C, representative 18F-FDG MicroPET/CT imaging of tumor-bearing mice. The tumors are indicated with arrows. Mice were fasted for 6 hours before detection. D, the ratios of the tumor SUVmax in the FBW7 group and the control group (n = 5; P < 0.05). E, expression of the rate-limiting enzymes GLUT1, GLUT4, HK2, LDHA, and LDHB decreased in tumors formed by FBW7 overexpression in SW1990 cells.

TXNIP is a target of FBW7 in PDAC

Next, to search for a possible molecular mechanism underlying the FBW7-mediated regulation of glucose metabolism, we used a high-throughput gene expression profiling array and found that a series of signaling pathways were altered by FBW7 overexpression (GEO: accession numbers GSE76443). Among the differentially expressed genes, FOXO1 and TXNIP are well-established regulators of glucose metabolism (26–28). Further validation using two cell lines indicated that FBW7 overexpression significantly altered TXNIP expression (Fig. 4A) but had little impact on FOXO1 expression (Supplementary Fig. S1C). We hypothesized that TXNIP was a potential effector of FBW7 in the regulation of glucose metabolism. To test this hypothesis, we examined the expression of TXNIP protein by immunoblotting after overexpression of FBW7 in two pancreatic cancer cell lines. TXNIP protein levels increased following FBW7 overexpression (Fig. 4B). On the contrary, downregulation of endogenous FBW7 by siRNA constructs significantly decreased the abundance of TXNIP in these two cells (Fig. 4C and D). Moreover, IHC staining for TXNIP was elevated in FBW7-overexpressing xenograft tumors (Fig. 4E). Next, we examined the correlation between FBW7 and TXNIP expression in tissues from PDAC patients and observed a positive correlation between FBW7 and TXNIP (Fig. 4F and Supplementary Table S3). Together, these data strongly suggest that TXNIP is a potential FBW7 target in pancreatic cancer.

TXNIP is a negative regulator of glucose metabolism

Although TXNIP is a negative regulator of glucose metabolism in many types of cancer cells, its role in pancreatic cancer has not been previously investigated. In the present study, we found that TXNIP expression is inversely correlated with the SUVmax value (Fig. 5A). To determine the impact of FBW7 expression on cellular metabolism, we generated PANC-1 and SW1990 stable cell lines ectopically expressing wild-type TXNIP (Fig. 5B). A subsequent analysis indicated that TXNIP inhibited glucose uptake and lactate production in PANC-1 and SW1990 cells (Fig. 5C and D). The ECAR and OCR results measured using the Seahorse metabolism analyzers further validated that TXNIP is a negative regulator of glycolysis and mitochondrial respiration (Fig. 5E and F). ATP production also decreased upon TXNIP overexpression (Fig. 5G). Furthermore, it was demonstrated that TXNIP decreased mitochondrial potential in PDAC cell lines (Fig. 5H). Accordingly, the expression of glycolytic enzymes related to glucose metabolism decreased dramatically after TXNIP overexpression (Fig. 5I and J; Supplementary Fig. S2A and S2B). These results confirm that TXNIP inhibits glucose metabolism in pancreatic cancer.

We then examined the influence of TXNIP on pancreatic cancer cell proliferation. As expected, a CCK8 proliferation assay indicated that TXNIP decreased the proliferation rate of PANC-1 and SW1990 cells (Fig. 5K). A subsequent colony-formation assay
demonstrated that TXNIP inhibited the colony-forming capacity of PDAC cancer cells (Supplementary Fig. S2C and S2D). Cell-cycle analyses indicated that overexpression of TXNIP inhibited cell-cycle progression and arrested the cell cycle at the G2–M phase (Supplementary Fig. S2E). In clinical specimens, we measured TXNIP expression using an IHC tissue microarray of PDAC samples and found that decreased TXNIP expression predicts a poor PDAC prognosis (Fig. 5L and Supplementary Fig. S2F and S2G). Finally, we silenced TXNIP in FBW7-overexpressing PANC-1 and SW1990 cells, and we found that TXNIP knockdown could reverse the effects of FBW7 overexpression in vitro, including proliferation and glucose metabolism inhibition (Supplementary Fig. S3). Thus, we believe that TXNIP is an important downstream effector of FBW7 in regulating glucose metabolism.

**FBW7 regulates TXNIP expression in a c-Myc-dependent manner.**

FBW7 is an E3 ubiquitin ligase and targets many substrates for proteasomal degradation. Among these substrates, HIF1α, PGC1α, and c-Myc are well-known regulators of metabolism. We previously reported that the expression of c-Myc decreased dramatically when FBW7 was overexpressed in pancreatic cancer (8). However, no change in the expression of PGC1α or HIF1α was observed upon FBW7 upregulation in pancreatic cancer. Therefore, we investigated the role of c-Myc in pancreatic cancer cell glucose metabolism and confirmed that c-Myc also promoted glucose metabolism in pancreatic cancer (Supplementary Fig. S4). To determine whether FBW7 regulates TXNIP through c-Myc, we first examined the TXNIP protein level in siRNA-transfected pancreatic cancer cells (Fig. 6A). The promoter region of TXNIP was reported to harbor E-box elements, which consist of the CAC(G/A)TG nucleotide sequence (27; Fig. 6B). We cloned the promoter region of TXNIP into the pGL3-basic vector and performed a dual luciferase assay to investigate whether c-Myc influences TXNIP promoter activity. The results indicated that cotransfection with c-Myc inhibited TXNIP promoter activity, whereas cotransfection with siRNA against c-Myc significantly increased TXNIP promoter activity (Fig. 6C). Moreover, c-Myc occupied the E-boxes in the TXNIP promoter region, as determined by ChIP assay (Fig. 6D). These findings suggest that c-Myc functions as a promoter of TXNIP transcription, which is consistent with observations from the study of triple-negative breast cancer (27).

To validate whether FBW7 regulated TXNIP expression in a c-Myc-dependent manner, we generated a dominant-negative FBW7 mutant, R465H, and designated it FBW7R465H. Compared with wild-type FBW7, FBW7R465H only marginally increased TXNIP expression (Fig. 6E and F). Consistent with this observation, TXNIP promoter activity and protein level both decreased with the expression of FBW7R465H (Fig. 6C). Furthermore, ChIP assay demonstrated that the introduction of FBW7 decreased c-Myc occupancy in the TXNIP promoter region, whereas FBW7R465H had little impact (Fig. 6H). Taken together, these results demonstrate that FBW7 inhibits glucose reprogramming in pancreatic cancer via the c-Myc/TXNIP axis. These results provide data regarding a novel function of FBW7 in PDAC glucose metabolism and indicate that FBW7 is a potential marker for pancreatic cancer diagnosis and prognosis and a target for pancreatic cancer treatment (Fig. 6I).
**Figure 5.**
TXNIP negatively regulates glucose metabolism and proliferation in pancreatic cancer. **A,** analysis of the SUV\(_{\text{max}}\) in TXNIP\(^{\text{low}}\) and TXNIP\(^{\text{high}}\) groups (n = 60; P < 0.05). **B,** overexpression of FLAG-tagged TXNIP in PANC-1 and SW1990 cells. **C,** TXNIP overexpression reduced the glucose uptake capacity of PDAC cells. **D,** lactate production was lower in the TXNIP-overexpressing PDAC cells. **E,** TXNIP negatively regulated the glycolysis rate, reflected by the ECAR. **F,** TXNIP inhibited the OCR. **G,** TXNIP decreased ATP production in PANC-1 and SW1990 cells. **H,** mitochondrial potential was decreased upon TXNIP overexpression. **I,** TXNIP led to changes in the expression of rate-limiting enzymes of the glycolysis cascade in PANC-1 cells. **J,** TXNIP led to changes in the expression of rate-limiting enzymes of the glycolysis cascade in SW1990 cells. **K,** TXNIP inhibited cell proliferation as measured by a CCK-8 proliferation kit. **L,** Kaplan–Meier analysis of the overall survival rate of patients with pancreatic cancer, according to TXNIP expression (n = 86; P < 0.001, log-rank test).
Discussion

Based on the observation that even in the presence of an oxygen supply, tumor cells preferentially use glycolysis over mitochondrial oxidative phosphorylation (OXPHOS) for glucose-dependent ATP production to fuel mitochondrial respiration, Otto Warburg put forward the notion of the "Warburg effect" in the 1920s (29). Advances in the understanding of the biology of tumor progression and metastasis have clearly highlighted the
importance of aberrant tumor metabolism. The manifestation of the Warburg effect in today's clinical setting is the use of 18F-FDG to detect tumors with an increased glucose uptake. The elevated uptake visualized by 18F-FDG PET/CT correlates with a poor prognosis and a higher metabolic burden in many types of tumors (23, 30).

Mounting evidence indicates that the reprogramming of tumor metabolism is controlled by various oncogenic signals (31, 32). In pancreatic cancer, the Ras oncoprotein has been shown to promote metabolic transformation (33–35). Although KRAS mutations were detected in >90% of PDAC patients and were proposed to be initiators of PDAC, KRAS remains an undruggable target. Elevated oncogenic KRAS activity stimulates many downstream signaling pathways (36–37). Therefore, strategies targeting the downstream effectors of KRAS might provide solutions to the inhibition of certain metabolic pathways. Our previous study reported that ERK activation caused by KRAS mutation in PDAC resulted in the destabilization of FBW7 (8). However, the specific role of FBW7 in PDAC remains unclear.

In the present study, we first provided clinical evidence that FBW7 expression affects glucose metabolism in PDAC with PET/CT data. We then used a series of aerobic glycolysis-related assays, including the examination of glucose uptake, lactate production, OCR, ECAR, and ATP production, and mitochondrial membrane potential. Overexpression of FBW7 dramatically inhibited glucose metabolism in PDAC cells. We confirmed these results in an in vivo xenograft model. All the enzymes related to glucose transportation (GLUT1, GLUT4, HK2, LDHA, and LDHB) decreased dramatically in the FBW7-overexpressing PDAC cells compared with the control cells. Given the important role of FBW7 in PDAC glucose metabolism, we further explored the potential underlying mechanism.

FBW7 has been reported to repress synthesis of cholesterol and fatty acids lipid homeostasis through modulating SREBP stability directly (38). However, the role of FBW7 in glucose transformation has seldom been studied. To investigate whether FBW7 could affect the downstream expression of glycolysis-related genes, we performed high-throughput screening to identify possible genes necessary for the coordinate regulation of FBW7-mediated glucose metabolism. Interestingly, the expression levels of many glycolysis-related genes were altered upon the upregulation of FBW7. Among the altered glycolysis-related genes in the database, we selected TXNIP as the target gene and investigated whether FBW7 regulates glycolysis via TXNIP in PDAC. TXNIP has been identified as a tumor suppressor gene in various solid tumors and hematologic malignancies (27). Moreover, recent evidence indicates that TXNIP also functions as a potent negative regulator of glucose uptake and aerobic glycolysis. We confirmed these results using q-PCR and Western blot analysis, which demonstrated that the mRNA and protein levels of TXNIP in FBW7-transfected PDAC cells were significantly higher than those in control cells, indicating that TXNIP was regulated by FBW7, predominantly via transcriptional modifications.

FBW7 has been reported to function as a tumor suppressor by targeting multiple oncoprotein substrates, such as cyclin E, c-Myc, c-Jun, PGC-1α, HIF1α, and Mcl-1, for degradation (6). Among the known substrates of FBW7, HIF1α, PGC-1α, and c-Myc have been reported to play critical roles in the regulation of metabolism (16, 39, and 17). These three substrates are also important transcription factors responsible for activating or repressing downstream individual genes. We previously found that c-Myc expression decreased dramatically upon FBW7 upregulation, whereas no reduction was observed in PGC-1α expression (8). Here, we again measured HIF1α levels and observed no alteration. c-Myc is a multifunctional transcription factor that drives the multiple synthetic functions necessary for rapid cell division and simultaneously inhibits the expression of genes with antiproliferative functions (40). Intriguingly, multiple studies have demonstrated that c-Myc can directly bind to the promoters of thousands of genes—up to 30% of all known genes (41). In addition, many of the metabolic changes that occur in transformed cells are driven by c-Myc overexpression (40). Thus, we proposed that FBW7 might regulate TXNIP in a c-Myc–dependent manner. To test this, we first investigated whether TXNIP expression changed in response to c-Myc downregulation. We found that c-Myc could suppress TXNIP promoter activity and inhibit TXNIP expression. These findings were consistent with a previous report that c-Myc could function as a promoter of TXNIP transcription. Finally, we validated that FBW7 regulated TXNIP expression in a c-Myc–dependent fashion by generating a FBW7 mutant that lost its E3 ligase activity.

In conclusion, we demonstrated a novel role of FBW7 in glucose metabolism in pancreatic cancer. Mechanistically, FBW7 regulates TXNIP expression in a c-Myc–dependent manner. Thus far, therapeutic strategies directly targeting KRAS or c-Myc have proven to be technically difficult. Therefore, alternative approaches that focus on interfering with c-Myc–mediated downstream effectors might provide novel therapeutic avenues for PDAC.

Disclosure of Potential Conflicts of Interest
No potential conflicts of interest were disclosed.

Authors’ Contributions
Conception and design: S. Ji, Y. Qin, J. Xu, Q. Ni, M. Li, X. Yu
Acquisition of data (provided animals, acquired and managed patients, provided facilities, etc.): S. Ji, Y. Qin, R. Huang, S. Shi, J. Liu, K. Jin, D. Liang, W. Xu, L. Liu, C. Liu, X. Yu
Analysis and interpretation of data (e.g., statistical analysis, biostatistics, computational analysis): C. Liang, M. Li, X. Yu
Writing, review, and/or revision of the manuscript: S. Ji, Y. Qin, J. Xu, Q. Ni, M. Li, X. Yu
Administrative, technical, or material support (i.e., reporting or organizing data, constructing databases): C. Liang, S. Shi, B. Zhang, J. Xu, X. Yu
Study supervision: X. Yu
Other (my lab provided some reagents from our unique GEMM): P.J. Chiao

Acknowledgments
The authors thank Huanyu Xia for assistance in collecting the patient data.

Grant Support
This work was supported by National Natural Science Foundation (81372631, 81201900, 81172276, and 81101565), Sino-German Center (GZ657), Ph.D. Programs Foundation of Ministry of Education of China (20120071120104), and Program of Science and Technology Commission of Shanghai (13431900105 and 13DZ1942802).

The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked advertisement in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

Received October 1, 2015; revised February 17, 2016; accepted March 7, 2016.

Published OnlineFirst March 16, 2016; DOI: 10.1158/1078-0432.CCR-15-2380

www.aacjrournals.org

Clin Cancer Res; 22(15) August 1, 2016 3959

Downloaded from clincancerres.aacjrournals.org on June 13, 2021. © 2016 American Association for Cancer Research.
References
FBW7 (F-box and WD Repeat Domain-Containing 7) Negatively Regulates Glucose Metabolism by Targeting the c-Myc/TXNIP (Thioredoxin-Binding Protein) Axis in Pancreatic Cancer

Shunrong Ji, Yi Qin, Chen Liang, et al.