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Original Article

FBP1 binds to the bromodomain of BRD4 to inhibit pancreatic cancer progression

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Abstract: Pancreatic ductal adenocarcinoma (PDAC) is a highly aggressive tumour that is characteristically unresponsive to most chemotherapeutic regimens. Bromodomain and extra terminal domain (BET) inhibitors that specifically repress the function of BET family proteins, such as BRD4, are under evaluation in clinical trials for their activity in repressing cancer growth. However, resistance to BET inhibitors has hindered their further clinical application in pancreatic cancer. We previously reported that FBP1 contributes to the resistance to BET inhibitors, but the underlying mechanism of this resistance remains unclear. Herein, we demonstrate that FBP1 is a binding partner of BRD4 in pancreatic cancer cells. We reveal that FBP1 binds to the BD2 domain of BD4 in an acetylation-dependent manner. Moreover, we found that Tip60 and HDAC3 were key to the acetylation and de-acetylation of FBP1 at K110 and K113, which are critical for mediating FBP1-BRD4 binding in pancreatic cancer cells. Furthermore, our data indicate that FBP1 decreases the expression of genes downstream of BRD4 to inhibit pancreatic cancer cell progression. Our results, therefore, provide evidence of the novel anti-tumour effect of FBP1 via its blockade of BRD4 function in pancreatic cancer cells.

Keywords: Fructose-1,6-biphosphatase (FBP1), bromodomain-containing protein 4 (BRD4), pancreatic cancer, acetylation

Introduction

Pancreatic ductal adenocarcinoma (PDAC) is a highly aggressive tumour that is characteristically unresponsive to most chemotherapeutic regimens [1]. Comprehensive studies on PDAC carcinogenesis over recent decades have identified a number of oncogenic proteins that contribute to pancreatic cancer progression [2]. The bromodomain and extra terminal domain (BET) family of proteins, including BRD2, BRD3, and BRD4, cooperate with RNA polymerase II to transcriptionally regulate gene expression and promote PDAC cell proliferation and metastasis [3]. Encouragingly, BET inhibitors, such as JQ1, can reportedly bind specifically to the bromodomain of BRD4 to disrupt the interaction between BRD4 and acetylated lysine residues in histones [4]. The JQ1 derivative is a promising anti-tumour agent that has been evaluated in clinical trials for different sorts of cancer [5]. Although current literature shows that JQ1 can inhibit pancreatic cancer cell growth, resistance to JQ1 has hindered its further clinical application in pancreatic cancer [5]. Therefore, exploring the underlying mechanism of the resistance to JQ1 could provide more therapeutic strategies to improve the survival of PDAC patients.

Fructose-1,6-biphosphatase (FBP1) converts fructose-1, 6-bisphosphate to fructose-6-phosphate and acts as a rate-limiting enzyme in gluconeogenesis [6]. The role of FBP1 in modulating glucose metabolism and blocking anaerobic glycolysis causes it to inhibit tumour cell progression in various types of malignant cancers [7-10]. Our group previously revealed that FBP1 inhibits the IQGAP1/MAPK/c-Myc pathway in pancreatic cancer [11] and contributes to the sensitivity of pancreatic cancer cells to

BET inhibitors [12], but the underlying mechanism has yet to be determined.

Given that BET inhibitors target the bromodomain of BRD4 specifically, we aimed to explore the potential relationship between FBP1 and BRD4. In this study, we demonstrated that FBP1 interacts with BRD4 in pancreatic cancer cells. We found that FBP1 bound to the BD2 domain of BRD4 in an acetylation-dependent manner. Moreover, Tip60 and HDAC3 were required for the acetylation or de-acetylation of FBP1 at K110 and K113, respectively, which are critical for mediating FBP1-BRD4 binding in pancreatic cancer cells. Furthermore, our data indicated that FBP1 decreases the expression of genes downstream of BRD4 to inhibit pancreatic cancer cell progression. Taken together, our results provide evidence of the novel anti-tumour effect of FBP1 via its blockade of BRD4 function in pancreatic cancer cells.

Methods and materials

Cell lines and cell culture

The human pancreatic cancer cell lines PANC-1 (#SCSP-535) and BxPC-3 (#TCHu 12) were purchased from the Chinese Academy of Science Cell Bank. All cells were cultured in Dulbecco's modified Eagle medium (DMEM) (Thermo Fisher Scientific, China) with 10% foetal bovine serum (FBS) (Thermo Fisher Scientific, China) supplemented with 5% CO_2 at 37°C.

Immunoprecipitation and Western blotting analysis

Human pancreatic cancer cells (PANC-1 and BxPC-3 cells) were washed with 1 × PBS and harvested from culture dishes. For immunoprecipitation experiments, cells were harvested and resuspended in 1 ml of RIPA buffer for 15 min. The cell lysate was centrifuged for 15 min at 13200 r/m at 4°C. The supernatant was incubated with Pierce Protein G Agarose and primary antibody or IgG in a cold room overnight. The beads were washed five times with IP buffer, resuspended with sample loading buffer and heated at 100°C for 5 min. The supernatant was used for further Western blotting.

For Western blotting, cells were lysed with 1 × RIPA buffer (Cell Signalling Technology) for 15 min on ice. The supernatant was obtained after centrifugation at 13200 r/min and quantified by using a BCA protein assay kit (cat no. PA115, Tiangen Biotech, Beijing, China) according to the manufacturer's protocol. Eighty micrograms of protein were subjected to separation by 8-10% SDS-PAGE and then transferred to a PVDF membrane. The membrane was washed with 1 × TBST and immersed in 5% non-fat milk at room temperature for 1 h. Next, the membrane was incubated with the corresponding primary antibodies at 4°C for more than 10 h. Then, the membrane was incubated with secondary antibody at room temperature for 1 h. Finally, target protein bands were visualized with a chemiluminescent western blot detection kit (cat no. 32209, Thermo Fisher Scientific, USA).

Antibodies, chemicals and plasmids

Antibodies against FBP1 (ab109732) (1:1000 for Western blotting), BRD4 (ab128874) (1:1000 for Western blotting), TWIST1 (ab50887) (1:1000 for Western blotting), Tip60 (ab23886) (1:1000 for Western blotting), HDAC3 (ab32369) (1:1000 for Western blotting), BRD2 (ab139690) (1:1000 for Western blotting)and GAPDH (ab8245) (1:5000 for Western blotting) were obtained from Abcam. Anti-BRD3 antibody (A302-368A) (1:1000 for Western blotting) was obtained from Bethyl Laboratories. JQ1, MG32 and TSA were obtained from Selleckchem (Houston, USA). GST-FBP1 was cloned into the pGEX-4T-1 backbone vector. FBP1 K110R/K113R, FBP1 K110Q/K113Q, and BD2 (YN/AA) mutant constructs and the GST-FBP1 construct were generated with a KOD-Plus-mutagenesis kit (Toyobo, Japan).

Glutathione S-transferase (GST) pull-down assay

Cells were lysed with 1 × RIPA buffer for 30 min at 4°C. GST fusion proteins were immobilized on glutathione-Sepharose beads (GE Healthcare Lifesciences, USA). After washing with lysis buffer, the beads were incubated with cell lysates for 4 h. The beads were then washed four times with binding buffer and resuspended in sample buffer. Bound proteins were subjected to SDS-PAGE and western blotting.

Real-time reverse transcription-PCR

Cells were washed with 1 × PBS and incubated with 1 ml of TRIzol reagent (Thermo Fisher

Scientific, USA) for 20 min on ice. RNA extracted with TRIzol reagent was subjected to reverse transcription to generate cDNA with a PrimeScript™ RT reagent kit (cat no. RR037A, Shigo, Japan). Then, real-time qRT-PCR was performed with a TB Green™ Fast qPCR Mix kit (cat no. RR430A, Shigo, Japan) with the cDNA used as a template. The -ΔΔCt method was used for quantification, and β-actin was used as a housekeeping gene. The following primers were used for RT-PCR: β-actin primers: forward, 5'-CCCTGGCTCCTAGCACCAT-3', and reverse, 5'-AGAGCCACCAATCCACACAGA-3'; WNT5a primers: forward, 5'-TACTCTGGGGACTTTGCAGG-3', and reverse, 5'-ACCTAGCGACCACCAAGAAT-3'; FLRT2 primers: forward, 5'-ATCTCCCAGGTACGCATCTG-3', and reverse, 5'-TGAGCCATTCTGTGACCCAT-3'; and PDE9A primers: forward, 5'-GACGCGATGTTCCCACTTAC-3', and reverse, 5'-CTGAGGGTGACAGGGTTGAT-3'.

Chromatin immunoprecipitation (ChIP) and ChIP-qPCR

ChIP was performed with a chromatin extraction kit (Abcam, ab117152, USA) and ChIP Kit Magnetic-One Step buffers (Abcam, ab156907, USA) following the manufacturer's instructions. Anti-BRD4 antibody (Cell Signalling Technology, 13440, USA, diluted 1:50) was used for the ChIP assay. Purified DNA was analysed by real-time PCR with a PCR kit (TaKaRa Bio, Inc., Japan) according to the manufacturer's protocols. The following primers were used: WNT5a ChIP primers: forward, 5'-CGGGCCACAGTTGAGTAGTG-3', and reverse, 5'-CGGTAATTAGGGCTTTCCAA-3'; FLRT2 ChIP primers: forward, 5'-CAATGGCACGATCTCAGCTC-3', and reverse, 5'-CATGCCTGTAATCCCAGCAC-3'; PDE9AChIP primers: forward, 5'-AAGAGGAGGGGAGAGAGGAG-3', and reverse, 5'-CTCAGCCAACCACAGCCG-3'.

RNA interference

Lentivirus vector-based short hairpin RNAs (shRNAs) (Sigma-Aldrich, USA) were transfected into 293T cells. After 72 h of transfection, 239T cell media containing viruses were harvested. To infect pancreatic cancer cells, the pancreatic cancer cell culture medium was replaced with the above viral media. After 72 h of infection and puromycin selection, pancreatic cancer cells were harvested for further experiments. The shRNA sequences were as follows: shTip60#1: 5'-CCGGCCGATATGTCAGGACCTAAATCTCGAGATTTAGGTCCTGACATATCGGTTTTTTG-3'; shTip60#2: 5'-CCGGTCGAATTGTTTGGGCACTGATCTCGAGATCAGTGCCCAAACAATTCGATTTTT-3'; shHDAC3: 5'-CCGGCCTTCCACAAATACGGAAATTCTCGAGAATTTCCGTATTTGTGGAAGGTTTTT-3'; shFBP1: 5'-CCGGCCACCATCAAATGCTGTAGAACTCGAGTTCTACAGCATTTGATGGTGGTTTTTG-3'; and shBRD4: 5'-CCGGCCTGGAGATGACATAGTCTTACTCGAGTAAGACTATGTCATCTCCAGGTTTTT-3'.

Cell proliferation assay

The pancreatic cancer cell growth rate was analysed by using the CCK8 assay. A total of 1000 cells were placed in each well of a 96-well plate, and the media were replaced with fresh media containing 10 μl of CCK8 reagent (#K1018, APExBIO) at the same time on the first, second, third and fourth day before incubation at 37° in 5% CO_2 for 1 h. The absorbance value was measured at a wavelength of 450 nm.

Generation of xenografts mouse model

Four- to five-week-old nude mice (Vitalriver, China) were randomly divided into shControl, shFBP1, shBRD4 and shFBP1 + shBRD4 groups (n=6/group). Pancreatic cancer cells (1×10^7) infected with shRNAs were subcutaneously injected into the left dorsal flanks of the mice. The tumour volume (length × (width^2)/2) was measured every other day for 21 days. The xenograft mouse model protocol was approved by the Institutional Animal Care and Use Committee of Tongji Medical College, Huazhong University of Science and Technology.

In vitro invasion assay

An in vitro cell invasion assay was performed using a BioCoat Matrigel invasion chamber (BD Biosciences) according to the manufacturer's protocol. PANC-1 and BxPC-3 cells were cultured in the insert of the chamber for 24 h. The cells were fixed in methanol for 15 min and then stained with 1 mg/ml crystal violet for 20 min. At least five fields for each group were photographed after staining, and invaded cells were counted.

Statistical analysis

All grouped data are presented as the means ± SDs. Comparisons between groups were calculated by one-way or two-way ANOVA using

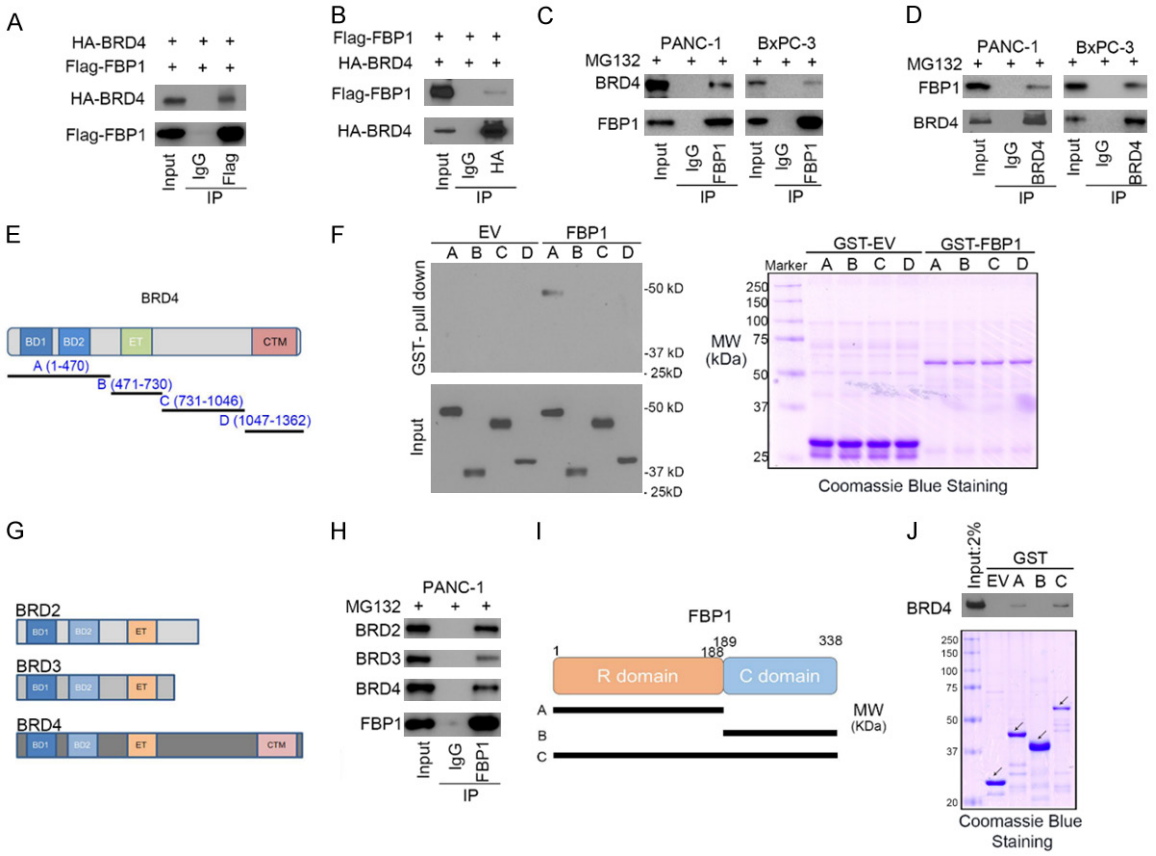

Figure 1. FBP1 interacts with BRD4 in pancreatic cancer cells. A. Western blotting analysis of WCLs (whole cell lysates) of 293T cells. The immunoblots (IB) are representative of results from three independent experiments. B. Western blotting analysis of WCLs of 293T cells. The immunoblots (IB) are representative of results from three independent experiments. C. Western blotting analysis of WCLs of PANC-1 and BxPC-3 cells. The cells were treated with 20 uM of MG132 before harvested for Western blotting analysis. D. Western blotting analysis of WCLs of PANC-1 and BxPC-3 cells. The cells were treated with 20 uM of MG132 before harvested for Western blotting analysis. E. Schematic depicting a set of BRD4 recombinant protein constructs. F. Western blotting analysis of Flag-tagged BRD4 recombinant proteins in PANC-1 WCL pulled down by GST or GST-FBP1 proteins. The right panel shows Coomassie Blue staining of GST and GST-FBP1 recombinant protein input. G. Schematic depicting the domain of BRD2, BRD3 and BRD4. H. Western blotting analysis of WCLs of PANC-1 cells. The cells were treated with 20 uM of MG132 before harvested for Western blotting analysis. I. Schematic depicting a set of FBP1 recombinant protein constructs. J. Western blotting analysis of BRD4 proteins in PANC-1 WCL pulled down by GST or GST-FBP1 recombinant proteins. The bottom panel shows Coomassie Blue staining of GST and GST-FBP1 recombinant protein input.

GraphPad Prism 5 software. Differences in which $P<0.05$ were considered statistically significant.

Results

FBP1 interacts with BRD4 in pancreatic cancer cells

FBP1 functions as a tumour suppressor in various types of cancers, such as pancreatic, breast, liver, and renal cancers [7, 8, 11, 13]. However, the mechanism through which FBP1 inhibits tumorigenesis has still not been explored. Our previous research found that the loss of FBP1 contributes to the resistance to BET inhibitors in pancreatic cancer cells [12]. Since BET inhibitors specifically repress the function of bromodomain-containing proteins, including BRD2, BRD3, and BRD4, we aimed to identify any potential direct interaction between FBP1 and these bromodomain-containing proteins. First, we found that ectopically expressed FBP1 and BRD4 bound to each other in 293T cells (**Figure 1A** and **1B**). Then, we studied the interaction between endogenous FBP1 and BRD4 in PANC-1 and BxPC-3 cells. Due to the low expression level of FBP1 in pancreatic

cancer [6], we pre-treated pancreatic cancer cells with the proteasome inhibitor, MG132, to increase endogenous FBP1 protein level (**Figure 1C** and **1D**). We showed that endogenous FBP1 interacted with BRD4 in pancreatic cancer cells (**Figure 1C** and **1D**).

To define the regions in BRD4 that interact with FBP1, we built recombinant Flag-tagged BRD4 protein constructs, as reported previously [14] (**Figure 1E**). GST pull-down assays demonstrated that the N-terminal region of BRD4 (amino acids 1-470) containing the BD1 and BD2 bromodomains interacts with FBP1 (**Figure 1F**). Because BRD2 and BRD3 contain similar BD1 and BD2 domains to those of BRD4, as indicated in **Figure 1G**, our results indicated further that FBP1 also bound to BRD2 and BRD3 in PANC-1 cells (**Figure 1H**).

To determine which region of FBP1 is responsible for its interaction with BRD4, we generated a recombinant GST-tagged FBP1 protein, as reported previously [6, 11] (**Figure 1I**). A GST pull-down assay revealed that the R domain of FBP1 (amino acids 1-188) mediates the interaction between FBP1 and BRD4 (**Figure 1J**). Taken together, our data suggest that FBP1 interacts with BRD4 in pancreatic cancer cells.

FBP1 binds to the BD2 domain of BRD4

Given that we found FBP1 to interact with the N-terminal region of BRD4 (**Figure 1F**) and bind with other bromodomain-containing proteins (BRD2 and BRD3) [14], we assumed that FBP1 binds to the bromodomain of BRD4. To test this hypothesis, FBP1 and BRD4 were overexpressed ectopically in 293T cells. The cells were treated with or without a BET inhibitor (JQ1). We have previously shown that JQ1 stabilizes FBP1 by disrupting the interaction between FBP1 and TRIM28 [12] and increases endogenous FBP1 [12] but not exogenous Flag-FBP1, except for co-transfection with TRIM28 plasmids (Supplementary Figure 1A and 1B), possibly due to the significantly overexpressed Flag-FBP1 in cells, which rendered JQ1's increase of FBP1 not observable. Our results revealed that treatment with JQ1 attenuated the interaction between FBP1 and BRD4 (**Figure 2A** and **2B**). To investigate the endogenous interaction between FBP1 and BRD4 and exclude the effect of JQ1's increase of FBP1, we pre-treated pancreatic cancer cells with the proteasome inhibitor, MG132, as indicated (**Figure 2C** and **2D**). Next, we treated PANC-1 cells with or without JQ1, and co-IP assays indicated that the administration of JQ1 decreased endogenous FBP1-BRD4 binding (**Figure 2C** and **2D**). In addition, FBP1 and recombinant BD1, BD2, or BD1+BD2 proteins were overexpressed in 293T cells (**Figure 2E** and **2F**). We found that FBP1 bound specifically to recombinant BD2 but not BD1 (**Figure 2E** and **2F**). Furthermore, mutations of conserved tyrosine and asparagine residues in the acetyl-lysine-binding pocket of BD2 to alanine ($BD2^{YN/AA}$) were generated, as previously reported [15]. Our findings demonstrated that $BD2^{YN/AA}$ pulled down less FBP1 in 293T cells than $BD2^{wt}$ did (**Figure 2G**). Hence, the BD2 domain of BRD4 is potentially responsible for FBP1-BRD4 interaction in pancreatic cancer cells (**Figure 2H**).

The acetylation of K110 and K113 in FBP1 is essential for FBP1-BRD4 interaction

Our findings up to this point reveal that the R domain of FBP1 mediates the binding of FBP1 to BRD4. To identify which specific subdomain of FBP1 interacts with BRD4, we generated GST-tagged FBP1 deletion constructs (Δ1-50, Δ51-100, Δ101-150, and Δ151-188) (**Figure 3A**). GST pull-down assays revealed that BRD4 bound to the region of FBP1 containing amino acids 101-150 (**Figure 3A**).

Reports show that the bromodomain of BRD4 recognizes the di-acetylation motif of target proteins, such as TWIST1 and Histone H4 [15]. Here, we analysed the amino acid sequence of FBP1 and found that K110 and K113 were highly similar to the di-acetylation motif of TWIST1 (**Figure 3B**). We also demonstrated that treatment with Trichostatin A (TSA) decreased the extent of the interaction between FBP1 and BRD4, suggesting that FBP1 binds to BRD4 in an acetylation-dependent manner (**Figure 3C**).

Next, we generated FBP1 K110R/K113R (FBP1-KR) and FBP1 K110Q/K113Q (FBP1-KQ) mutants to mimic de-acetylated and acetylated FBP1, respectively. We found that FBP1-KQ pulled down the most BRD4 in PANC-1 cells, while FBP1-KR pulled down the least BRD4 (**Figure 3D** and **3E**). Thus, our results indicate that the di-acetylation of K110 and K113 in FBP1 is essential for FBP1-BRD4 interaction.

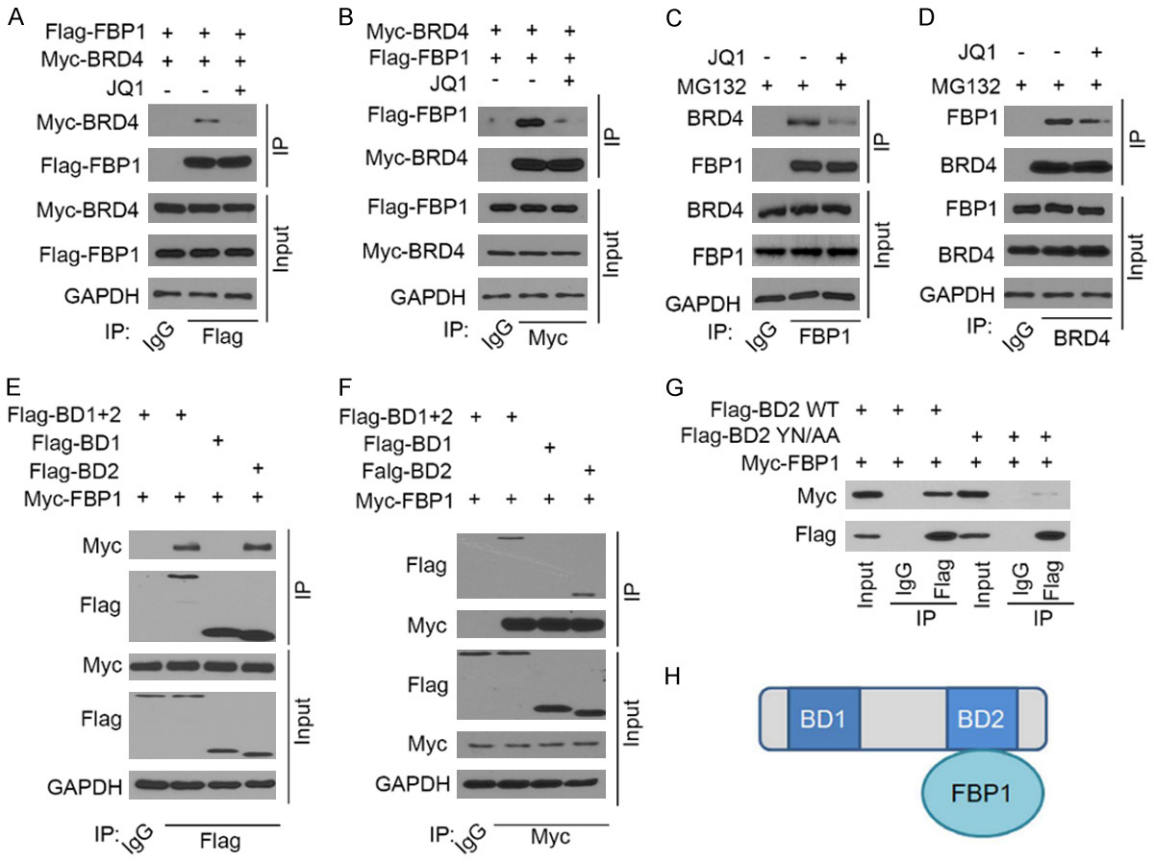

Figure 2. FBP1 binds the BD2 domain of BRD4. A and B. PANC-1 cells were transfected with indicated plasmids. The WCL of PANC-1 cells were subjected to Western blotting analysis. C and D. PANC-1 cells were treated with or without JQ1 (10 uM) for 24 h. The WCL of PANC-1 cells were subjected to Western blotting analysis after treated with 20 uM of MG132. E. Western blotting analysis of WCLs of 293T cells transfected with indicated constructs. F. Western blotting analysis of WCLs of 293T cells transfected with indicated constructs. G. Western blotting analysis of WCLs of 293T cells transfected with indicated constructs. H. Schematic depicting FBP1 interacted with BD2 domain, but not BD1 domain of BRD4.

Tip60 and HDAC3 modulate the di-acetylation of FBP1 in pancreatic cancer cells

Tip60 plays a key role in modulating the acetylation of TWIST1 in breast cancer cells [15]. To test whether FBP1 is regulated by Tip60 in pancreatic cancer, a co-IP assay was performed on PANC-1 cells and revealed that Tip60 interacted with FBP1 in PANC-1 cells (**Figure 4A** and **4B**). Subsequently, Tip60 was knocked down with a Tip60-specific short hairpin RNA (shRNA) that decreased FBP1-BRD4 binding in PANC-1 cells (**Figure 4C**). In contrast, the overexpression of wild-type HA-tagged Tip60 (HA-Tip60 WT), but not an enzyme-dead Tip60 mutant (HA-Tip60 ΔHAT), enhanced the interaction between FBP1 and BRD4 in PANC-1 cells (**Figure 4D**). We also showed that an ectopically overexpressed recombinant WT Tip60 protein increased FBP1 WT-BRD4 binding but not FBP1-KR-BRD4 binding in PANC-1 cells, indicating that Tip60 is critical for regulating the di-acetylation of FBP1 and promoting the interaction between FBP1 and BRD4 in pancreatic cancer cells (**Figure 4E** and **4J**).

We observed the interaction between endogenous FBP1 and HDAC3 in PANC-1 cells (**Figure 4F** and **4G**). To determine whether HDAC3 regulates the acetylation status of FBP1 and the interaction between FBP1 and BRD4 in pancreatic cancer cells, we knocked down HDAC3 using shRNA in PANC-1 cells. HDAC3 inhibition increased FBP1-BRD4 binding (**Figure 4H**). In contrast, HDAC3 overexpression reduced FBP1-WT-BRD4 binding but did not have any substantial effect on FBP1-KR-BRD4 interaction in PANC-1 cells (**Figure 4I**). For that

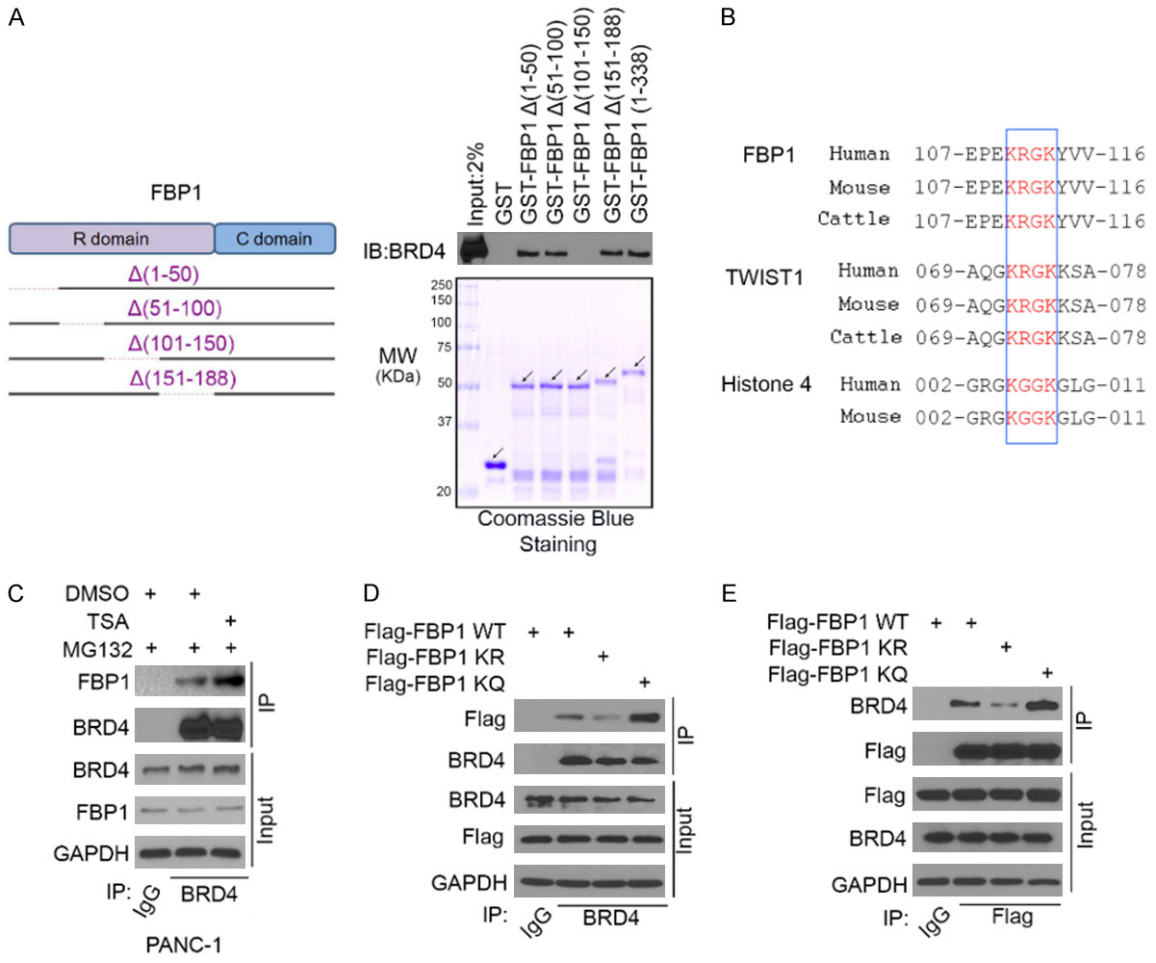

Figure 3. The acetylation of K110 and K113 in FBP1 is essential for the FBP1-BRD4 interaction. A. Schematic depicting a set of FBP1 recombinant protein constructs. Western blotting analysis of BRD4 proteins in PANC-1 WCL pulled down by GST or GST-FBP1 proteins. B. Schematic depicting a consensus "KRGK" amino acids sequence of FBP1. C. PANC-1 cells were treated with or without TSA for 24 h. After treating with 20 uM of MG132 with other 8 h, the WCL of PANC-1 cells were subjected to Western blotting analysis. D. Western blotting analysis of WCLs of PANC-1 cells transfected with indicated constructs. E. Western blotting analysis of WCLs of PANC-1 cells transfected with indicated constructs.

reason, our data indicate that HDAC3 might deacetylate FBP1 and block the binding of FBP1 to BRD4 (**Figure 4J**).

FBP1 decreases gene expression downstream of BRD4 in pancreatic cancer cells

FBP1 and TWIST1 bind to the same region of BRD4 (BD2) [15]. Our results demonstrated further that overexpressed FBP1-WT, but not FBP1-KR, competed with TWIST1 to bind to BRD4 in PANC-1 cells (**Figure 5A**). In contrast, FBP1 repression enhanced TWIST1-BRD4 interaction, as determined by assessing the acetylation status of FBP1 in PANC-1 cells (**Figure 5B**).

TWIST1 reportedly binds to BRD4 and transcriptionally regulates the expression of downstream genes *WNT5a*, *FLRT2,* and *PDE9A* [15]. We next sought to know if FBP1 regulates the expression of these genes via BRD4 in pancreatic cancer cells. Our findings established that FBP1 knockdown markedly increased BRD4 binding to the promoters of these genes in PANC-1 cells (**Figure 5C**). In the meantime, FBP1 inhibition upregulated *WNT5a*, *FLRT2,* and *PDE9A* expressions in PANC-1 cells, and FBP1-WT, but not mutant FBP1-KR, reversed these changes (**Figure 5D**). Additionally, the overexpression of TWIST1 increased *WNT5a*, *FLRT2*, and *PDE9A* expressions in PANC-1 cells,

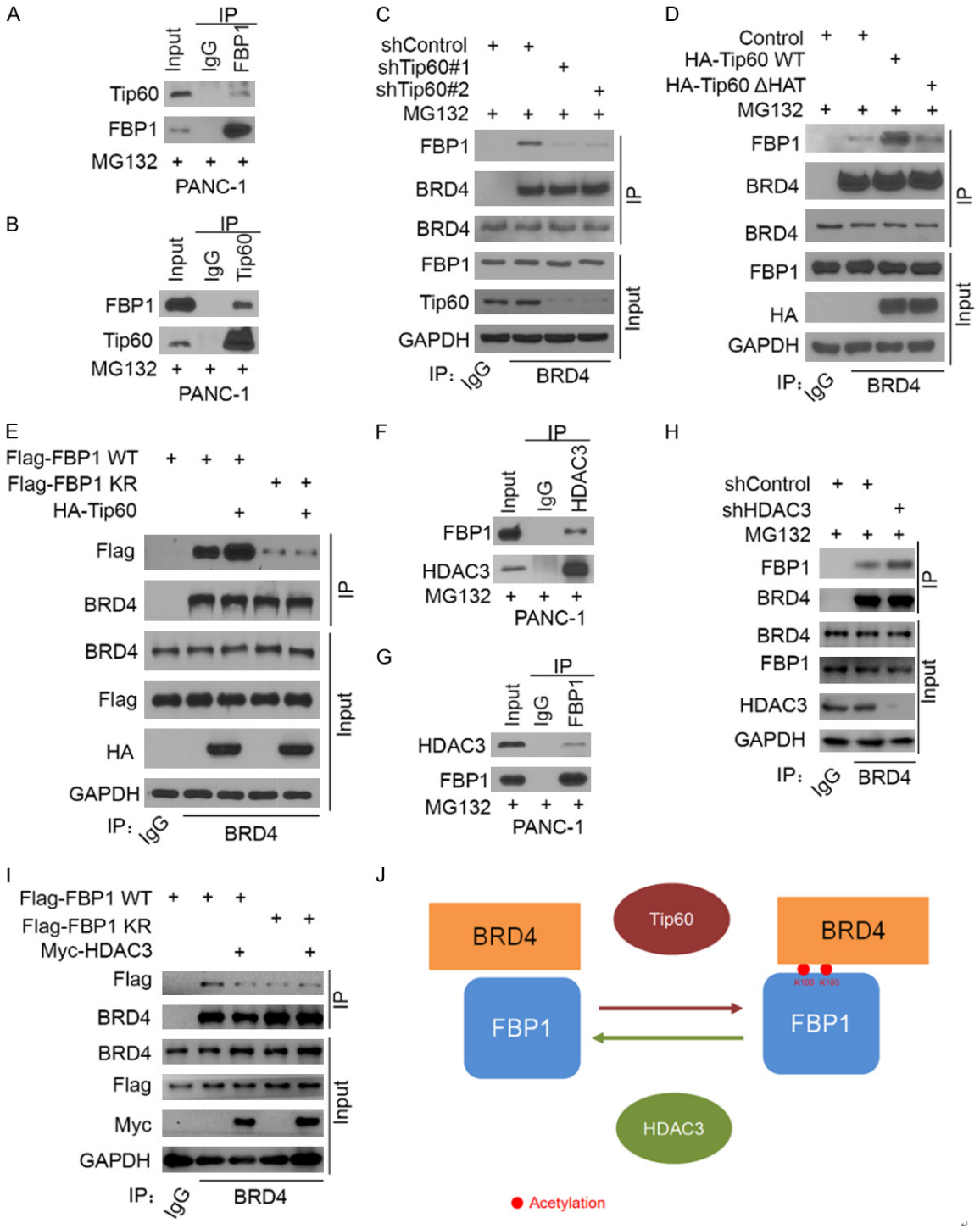

Figure 4. Tip60 and HDAC3 modulate the di-acetylation of FBP1 in pancreatic cancer cells. A. Western blotting analysis of WCL of PANC-1 cells. The cells were treated with 20 uM of MG132 before harvested for Western blotting analysis. B. Western blotting analysis of WCL of PANC-1 cells after treating with 20 uM of MG132 with other 8 h. C. Western blotting analysis of WCL of PANC-1 cells infected with indicated shRNAs. The cells were treated with 20 uM of MG132 before harvested for Western blotting analysis. D. Western blotting analysis of WCL of PANC-1 cells transfected with indicated plasmids. E. Western blotting analysis of WCL of PANC-1 cells transfected with indicated plasmids. F. Western blotting analysis of WCL of PANC-1 cells after treating with 20 uM of MG132 with other 8 h. G. Western blotting analysis of WCL of PANC-1 cells after treating with 20 uM of MG132 with other 8 h. H. Western blotting analysis of WCL of PANC-1 cells infected with indicated shRNAs. The cells were treated with 20 uM of MG132 before harvested for Western blotting analysis. I. Western blotting analysis of WCL of PANC-1 cells transfected with indicated plasmids. J. Schematic depicting that Tip60 and HDAC3 modulates the di-acetylation of FBP1 in pancreatic cancer cells.

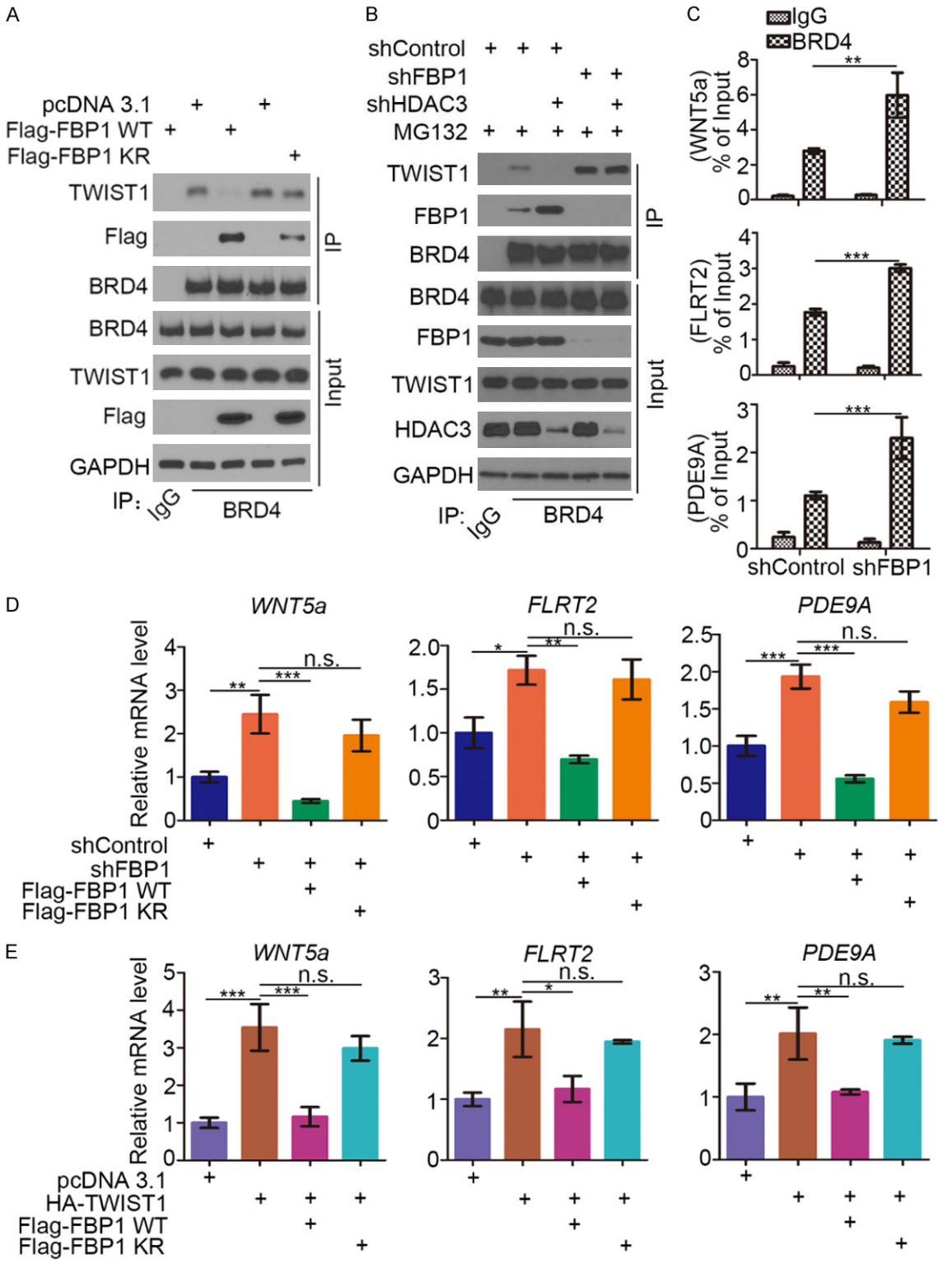

Figure 5. FBP1 decreases BRD4 downstream gene expression in pancreatic cancer cells. A. Western blotting analysis of WCL of PANC-1 cells transfected with indicated plasmids. B. Western blotting analysis of WCL of PANC-1 cells infected with indicated shRNAs. The cells were treated with 20 uM of MG132 before harvested for Western blotting analysis. C. PANC-1 cells were infected with indicated shRNAs. Cells were harvested for ChIP-qPCR analysis. Data presented as Means ± SD (n=3). **, P<0.01; ***, P<0.001. D. PANC-1 cells were transfected with shControl or shFBP1 for 48 h. Then, cells were transfected with indicated plasmids for 24 h. Cells were harvested for RT-qPCR

analysis. Data presented as Means ± SD (n=3). n.s., not significant; *, P<0.05; **, P<0.01; ***, P<0.001. E. PANC-1 cells were transfected with indicated plasmids for 24 h. Cells were harvested for RT-qPCR analysis. Data presented as Means ± SD (n=3). n.s., not significant; *, P<0.05; **, P<0.01; ***, P<0.001.

and the ectopic expression of FBP1-WT, but not mutant FBP1-KR, reversed this action (**Figure 5E**). In conclusion, we demonstrated that FBP1 decreases gene expression downstream of BRD4 in pancreatic cancer cells.

FBP1 inhibits pancreatic cancer progression partially through BRD4

Given that WNT5a contributes to the promotion of pancreatic cancer cell proliferation, epithelial-to-mesenchymal transition, and modulation of cell cycle progression [16-18], we examined FBP1's ability to inhibit the aggressive phenotype of pancreatic cancer through BRD4-WNT5a signalling. Our results showed that knocking down FBP1 promoted PANC-1 and BxPC-3 cell proliferation, which was halted by simultaneous BRD4 repression *in vitro* (**Figure 6A-D**).

A xenograft assay was also employed to determine the anti-tumour effect of FBP1 *in vivo*, and the results revealed that FBP1 inhibition led to increased tumour growth in nude mice (**Figure 6E-G**). However, the simultaneous co-knockdown of FBP1 and BRD4 attenuated the tumour growth-promoting effect of FBP1 knockdown alone (**Figure 6E-G**). Additionally, we determined that knocking down FBP1 increased the invasive ability of PANC-1 and BxPC3 cells (**Figure 6H** and **6I**). Similarly, the co-knockdown of FBP1 and BRD4 weakened this effect (**Figure 6H** and **6I**). These results suggest that FBP1 inhibits cancer cell progression in pancreatic cancer through BRD4.

Discussions

FBP1 expression is lost or downregulated in various types of malignant cancers, including liver cancer, breast cancer, non-small cell lung cancer, prostate cancer, and pancreatic cancer [8, 9, 11, 19]. We previously reported that the loss of FBP1 was closely associated with an unfavourable prognosis in pancreatic cancer patients [11]. In addition to modulating glucose metabolism to inhibit cancer cell proliferation, FBP1 can suppress tumour cell progression in an enzyme-independent manner [13]. The nuclear portion of FBP1 reportedly binds to HIF-1a to oppose renal carcinoma progression [13]. Also, FBP1 competes with ERK1/2 to bind to the WW domain of IQGAP1 and represses the MAPK pathway activation in pancreatic cancer cells [11]. Moreover, FBP1 is a negative regulator of the Wnt/β-Catenin pathway in breast cancer [20]. Thus, exploring the enzyme-independent role of FBP1 could lead to a better understanding of the anti-tumour mechanism of FBP1 in cancer cells. Here, we demonstrated that the di-acetylation of FBP1 at Thr^{110} and Thr^{113} mediates FBP1-BRD4 interaction, which suggests that FBP1 functions as a novel regulator of BRD4 in pancreatic cancer cells. Consistent with the findings of Li K et al, our data indicate that FBP1 could suppress the Wnt pathway by downregulating WNT5a expression in a BRD4-dependent manner.

BRD4 recognizes Histone H4 di-acetylated at K5 and K10 and recruits P-TEFb and RNA-Pol II to activate gene expression [3, 21]. The BD1 and BD2 domains of BRD4 are critical to its co-activation function [22]. Both domains contain a pocket motif used to recognize acetylated lysine amino acids in target proteins [15]. Interestingly, according to published literature and our results, there is no unanimous consensus on the amino acid sequences identified by BD1 and BD2. BD1 reportedly binds to Histone H4 [15] and ERG [22], amino acid sequences that contain a "KGGK" motif. In contrast, TWIST1 [15] and FBP1 interact with the BD2 domain of BRD4 through a conserved "KRGK" motif. In this study, we found that FBP1 inhibits WNT5a expression in pancreatic cancer cells, whereas TWIST1 promotes WNT5a expression in breast cancer cells. c-Myc is the best-studied downstream gene of BRD4. However, cDNA microarray analysis conducted previously by Shi J et al revealed that the BD2 domain does not regulate c-Myc expression [15]. In contrast, AR binds to the BD1 domain of BRD4 and increases c-Myc expression in prostate cancer cells [23]. The BD1 and BD2 domains, therefore, appear to perform different cellular functions.

Collectively, our data indicate that FBP1 interacts with BRD4 in pancreatic cancer cells. The di-acetylation of FBP1 at Thr^{110} and Thr^{113} was essential for FBP1-BRD4 interaction. We also

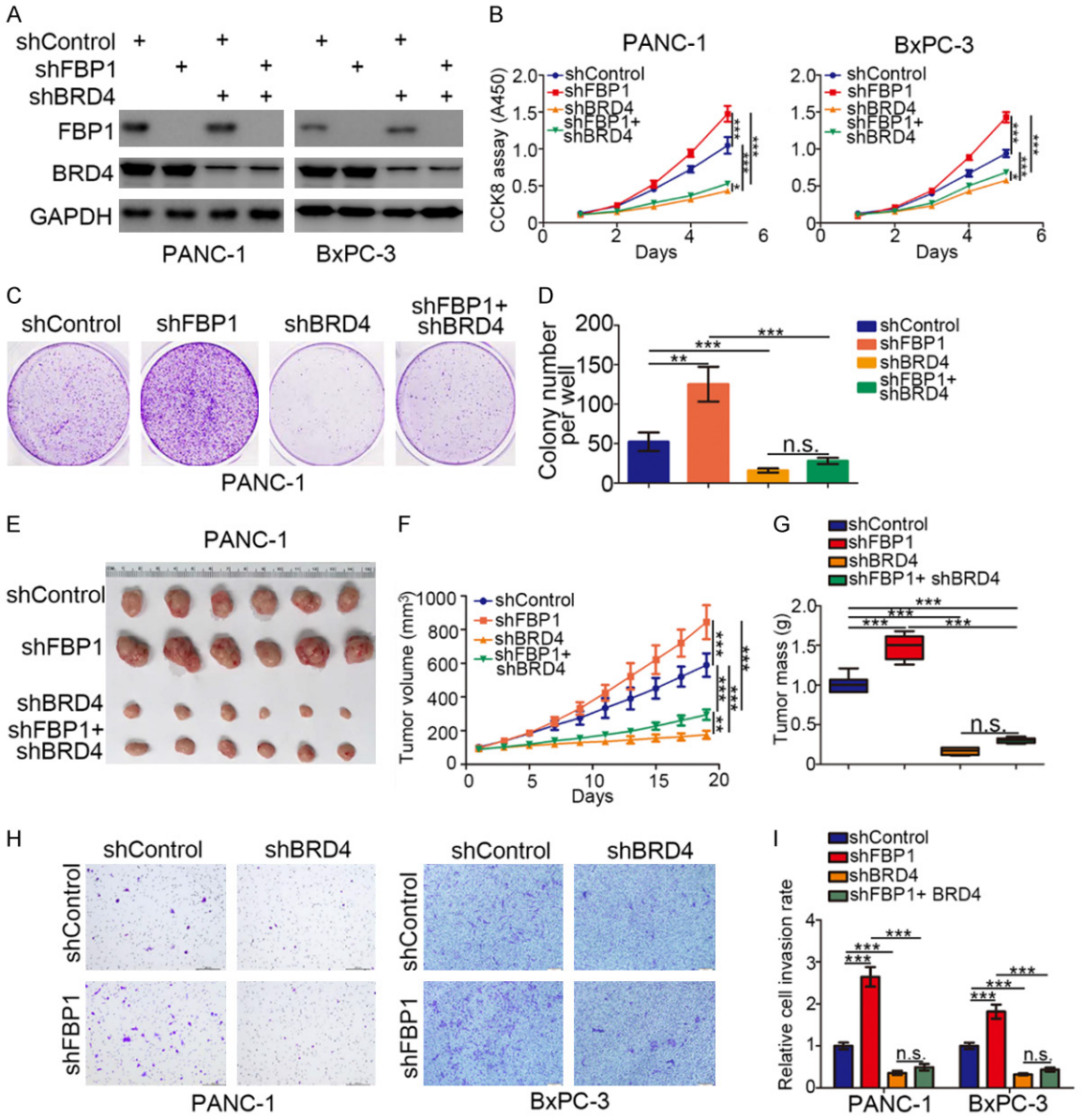

Figure 6. FBP1 inhibits pancreatic cancer progression partially through BRD4. (A-D) PANC-1 and BxPC-3 cells were infected with indicated shRNAs for 72 h. Cells were harvested for Western blotting analysis (A), CCK8 assay (B) and colony formation assay (C and D). Data presented as Means ± SD (n=3). n.s., not significant; *, P<0.05; **, P<0.01; ***, P<0.001. (E-G) PANC-1 cells were infected with indicated shRNAs for 72 h. Cells were harvested for xenografts assay. The tumor growth curve (F) and excised tumor mass (G) as indicated. Data presented as Means ± SD (n=6). n.s., not significant; *, P<0.05; **, P<0.01; ***, P<0.001. (H and I) PANC-1 and BxPC-3 cells were infected with indicated shRNAs for 72 h. Cells were harvested for in vitro invasion assay. Data presented as Means ± SD (n=3). n.s., not significant; ***, P<0.001.

established that Tip60 and HDAC3 modulated the di-acetylation of FBP1 in pancreatic cancer cells. Finally, we demonstrated that FBP1 inhibits pancreatic cancer progression partially through BRD4 by downregulating BRD4 target gene expression. Therefore, FBP1 is a negative regulator of BRD4, and the restoration of FBP1 expression might serve as a novel strategy for pancreatic cancer treatment.

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Disclosure of conflict of interest

None.

Abbreviations

FBP1, Fructose-1,6-biphosphatase; BRD4, Bomodomain-containing protein 4; ChIP, Chromatin immunoprecipitation; Co-IP, co-immunoprecipitation; GST, Glutathione S-transferase; TSA, Trichostatin A.

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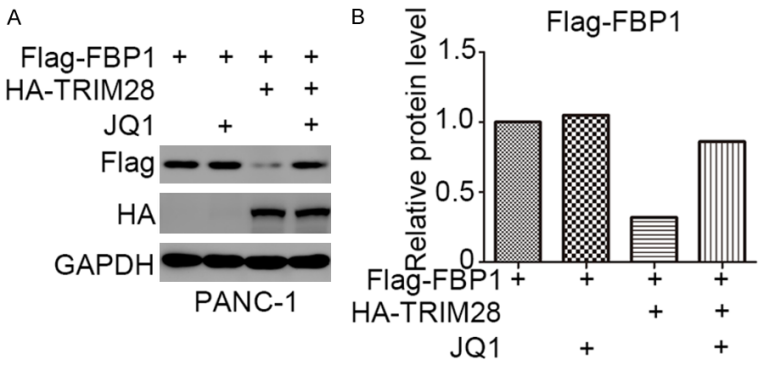

Supplementary Figure 1. PANC-1 cells were transfected with indicated plasmids for 24 h. The cells were treated with 5 uM of JQ1 for 24 h before harvested for Western blotting analysis (A). The protein expression level of Flag-FBP1 was normalized to the protein level of GAPDH and then to the value in Flag-FBP1 transfection alone group (B).