原著

ORIGINAL

Analysis of the anti-tumor mechanism of BRD4 inhibition in hepatocellular carcinoma

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ABSTRACT

　　Bromodomain and extra terminal (BET) family proteins, which include BRD4, are readers of histone acetyl-lysines and key regulators of gene transcription. BRD4 inhibitors exert anti-tumor effects in various cancers, including hepatocellular carcinoma (HCC). We investigated the mechanism underlying the anti-tumor effects of BRD4 inhibition in HCC. We first tested the effects of the BRD4 inhibitor JQ1 in a series of 9 HCC cell lines and found that it strongly suppressed HCC cell proliferation by inducing cell cycle arrest and apoptosis. Gene expression microarray analysis revealed that JQ1 also induced marked changes in the gene expression profiles of HCC cells, and genes associated with cell cycle and apoptosis were significantly enriched among the affected genes. Notably, a number of cancer-related genes, including BCAT1, DDR1, GDF15, FANCD2, SENP1 and TYRO3, were strongly suppressed by JQ1 in HCC cells. We also confirmed BRD4 bound within the promoter regions of these genes, which suggests they are targets of BRD4 in HCC cells. JQ1 thus appears to exert its anti-tumor effects in HCC by suppressing multiple BRD4 target genes.

1.　Introduction

　　Hepatocellular carcinoma (HCC) is one of the most commonly occurring cancers around the world, with approximately 782,000 new cases and 723,000 deaths annually [1)]. HCC is associated with specific risk factors, including hepatitis B or C virus infection, high alcohol intake and nonalcoholic fatty liver disease (NAFLD), and many HCCs arise in the context of chronic hepatitis [2)]. Despite recent improvements in the clinical treatments for HCC, it remains a dismal disease with a poor prognosis, and development of a new therapeutic strategy is much needed.

　　Recent studies have shown that a wide variety of epigenetic abnormalities, including aberrant DNA methylation and histone modifications, are involved in the development of cancer [3)]. These epigenetic alterations are considered to be potential molecular targets for cancer diagnosis and promising therapies, and a number of

drugs targeting epigenetic alterations have been developed [3)]. The effectiveness of epigenetic therapy has been particularly evident in hematological malignancies, though recent studies are beginning to show its usefulness in solid tumors [3)]. Epigenetic alterations are strongly implicated in the development and progression of HCC, making them potential therapeutic targets [4)]. However, much about the clinical usefulness of epigenetic therapy in HCC remains unclear.

　　Histone acetylation is a crucial epigenetic mechanism involved in regulating gene expression. Bromodomain and extra terminal (BET) family proteins recognize acetylated histones and play an essential role in transcriptional regulation. Members of BET family include BRD2, BRD3, BRD4, and BRDT [3)]. Among them, BRD4 is well known to maintain and facilitate oncogenic transcription directly by recruiting transcriptional machinery or indirectly by binding to enhancers, thereby contributing to cancer cell proliferation and survival [5, 6)]. The BET

inhibitor JQ1 competitively binds to acetyl-lysine recognition motifs and inhibits BRD4[7]. It also exerts strong anti-tumor effects in part through inhibition of MYC, which is well documented to be a major anti-tumor mechanism related to BRD4 inhibition[8]. However, BRD4 also targets other genes in cancer[9, 10]. In the context of HCC, oncogenes reportedly targeted by BRD4 include MYC and E2F2, but the anti-tumor mechanism of BRD4 inhibition in HCC is not fully understood[11, 12]. In the present study, we aimed to clarify the anti-tumor mechanism of BRD4 inhibition in HCC. To that end, we assessed the effect of the BRD4 inhibitor JQ1 on gene expression profiles in a series of HCC cell lines, which enabled us to identify novel BRD4 target genes in HCC.

2.   Materials and methods

2.1 Cell lines and tissue samples

HCC cell lines (JHH-4, Li-7, HepG2, Hep3B, HLE, HLF, huH-1, HuH-7 and PLC/PRF/5) were obtained and cultured as described previously[13]. Total RNA was isolated using TRIzol reagent (Thermo Fisher Scientific, Waltham, MA, USA). Total RNA from normal liver tissues from a healthy individual was purchased from BioChain (Hayward, CA, USA).

2.2 Cell viability assay

HCC cells were seeded into 96-well plates to a density of 3000 cells/well in 100 µl of medium. Cells were then treated with 0.001 µM, 0.048 µM, 0.24 µM, 1.2 µM, 6 µM or 30 µM JQ1 (Cayman Chemical Company, Ann Arbor, MI, USA). After incubation for 5 days, cell viability was assessed using a Cell Counting kit-8 (Dojindo, Kumamoto, Japan) according to the manufacturer's instructions.

2.3 Cell cycle and apoptosis assays

For cell cycle analysis, HCC cells were treated for 48 h with 1 µM JQ1 or DMSO, after which they were stained with propidium iodide (PI; Dojindo, Kumamoto, Japan) or with PI and an ApoScreen Annexin V Apoptosis Kit (Southern Biotech, Birmingham, AL, USA) according to the manufacturer's instructions. Flow cytometric analysis was then performed using a BD FACSCant II (BD Biosciences, Franklin Lakes, NJ, USA) and BD FACSDiva software (BD Biosciences). Data were analyzed using FlowJo software version 10 (FlowJo LLC, Ashland, OR, USA).

2.4 Quantitative reverse transcription-PCR

Single-stranded cDNA was prepared using PrimeScript RT Master Mix (Takara Bio Inc., Kusatsu, Japan). Quantitative reverse transcription-PCR (qRT-PCR) was carried out using PowerUp SYBR Green Master Mix (Thermo Fisher Scientific) and a 7500 Fast Real-Time PCR system (Thermo Fisher Scientific). β-actin (ACTB) was used as an endogenous control. Primer sequences are listed in Table 1.

2.5 Gene expression microarray analysis

HCC cells were treated for 24 h with 1 µM JQ1 or DMSO, after which total RNA was extracted using TRIzol reagent (Thermo Fisher Scientific). Gene expression microarray analysis was performed as described previously[13]. Briefly, 100 ng of total RNA were labeled using a Low-input Quick Amp Labelling kit One-color (Agilent Technologies, Santa Clara, CA, USA), after which the synthesized cRNA was hybridized to a SurePrint G3 Human GE microarray v2 (G4851, Agilent Technologies). The microarray data were analyzed using GeneSpring GX version 13 (Agilent Technologies).

2.6 Analysis using publically available datasets

Gene expression and survival data of primary HCC patients in The Cancer Genome Atlas (TCGA) network study were obtained from University of California, Santa Cruz (UCSC) Cancer Browser (https://xena.ucsc.edu/)[14]. Data of acetylated histone H3 lysine 9 and 27 in HepG2 cells were obtained from Encyclopedia of DNA Elements (ENCODE) database (https://genome.ucsc.edu/).

2.7 RNA interference

HCC cells (5×10^5 cells/well in 6-well plates) were transfected with 25 pmol of a Silencer Select siRNA targeting BRD4 (ID, s23901; Thermo Fisher Scientific) or a Silencer Select Negative Control (Thermo Fisher Scientific) using Lipofectamine RNAiMAX (Thermo Fisher Scientific) according to the manufacturer's instructions. After incubation for 48 h, cells were harvested and analyzed.

2.8 Chromatin immunoprecipitation

Chromatin immunoprecipitation (ChIP) was performed using a ChIP-IT High Sensitivity Kit (Active Motif, Carlsbad, CA, USA) according to the manufacturer's instructions. Briefly, 1×10^7 cells were fixed, sonicated and immunoprecipitated using a rabbit anti-BRD4 monoclonal antibody (#13440, Cell Signaling Technology,

Danvers, MA, USA) or control IgG. Input DNA and the immunoprecipitates were then subjected to quantita¬tive PCR analysis using PowerUp SYBR Green Master Mix (Thermo Fisher Scientific) according to the manufacturer's instructions. Primer sequences are listed in Table 1.

2.9 Statistical analysis

Quantitative variables were analyzed using Student's t test. Survival was analyzed using the log-rank test for 2-group comparisons. Values of P < 0.05 (2 sided) were considered statistically significant. Statistical analyses were carried out using EZR version 1.32 (Saitama Medical Center, Jichi Medical University, Saitama, Japan) [15].

3. Results

3.1 Anti-tumor effects of BRD4 inhibition in HCC cell lines

To evaluate the anti-tumor effects of BRD4 inhibition in HCC cells, we treated a series of 9 HCC cell lines with serial dilutions of JQ1 for 5 days and then performed cell viability assays. We found that JQ1 dose dependently suppressed proliferation of all the HCC cells tested (Figure 1). HuH-7, Hep3B and HLE cells were highly sensitive to JQ1, with IC50 values of 0.046, 0.048 and 0.076 μM, respectively (Figure 1A). Cell cycle analysis using flow cytometry revealed that treatment with JQ1 (1.0 μM for 48 h) led to increases

Table 1. Sequences of the primers used in this study

|	Forward	Reverse	Product size
qRT-PCR			
ACTB	5'-GCCAACCGCGAGAAGATGA-3'	5'-AGCACAGCCTGGATAGCAAC-3'	80 bp
BCAT1	5'-TTGGAACTGAGCCTTCTCTTGG-3'	5'-CACTGGGCTCAAGAGTACAAAG-3'	71 bp
BRD4	5'-CCGATTGATGTTCTCCAACTGC -3'	5'-TCTTGGCAAAGCGCATTTCG-3'	104 bp
DDR1	5'-AGGAGTTCTCCCGGAGCTAC-3'	5'-AAGGTCCTTCAGCACCACTC-3'	131 bp
E2F2	5'-ATCATGGAGCCCACAGCATC-3'	5'-GGCTGTCAGTAGCCTCCAA-3'	100 bp
FANCD2	5'-TGGGATTATTGGTGCTGTGACC-3'	5'-TGGCTCTCTCTTGGGTCAAAC-3'	83 bp
GDF15	5'-AGAGCTGGGAAGATTCGAACAC-3'	5'-AGATACGCAGGTGCAGGTG-3'	105 bp
MAPK3	5'-CATGAGAATGTCATCGGCATCC-3'	5'-GCAGATATGGTCATTGCTCAGC-3'	144 bp
MYC	5'-TTCTCTGAAAGGCTCTCCTTG-3'	5'-GTTGGTGAAGCTAACGTTGAG-3'	107 bp
NUAK1	5'-GACATGGTTCACATCAGACGAG-3'	5'-CCCTTTGCTGGCATATTCCATG-3'	129 bp
PAK1	5'-TCTAAACCTCTGCCTCCAAACC-3'	5'-AAATCTCTGGCCGCTCTTTC-3'	115 bp
SENP1	5'-AGAGGCGACATGTTAGTACAGC-3'	5'-AAATGTGTGGTGGGTTTGGC-3'	121 bp
TYRO3	5'-AAACAGCCTCCGGAGTGTATG-3'	5'-TGTTCTCCAGTTCCATTCGC-3'	146 bp
ChIP-qPCR			
BCAT1 #1	5'-TCCGTTCCCAAAAGCGAATG-3'	5'-TTATATCCCCCGCAAACGTCTC-3'	141 bp
BCAT1 #2	5'-AGACGTTTGCGGGGGATATAAG -3'	5'-ATGTGTGCGTGAATCGAACC-3'	100 bp
BCAT1 #3	5'-CAGGAAAGCGCATTTACAGC -3'	5'-TCCCCTGCTTGTGAAGTTTG-3'	122 bp
DDR1 #1	5'-AGGAGCAAAGGTTGGTGGAG-3'	5'-CCAGAGAAGGGAGCAGAGC-3'	89 bp
DDR1 #2	5'-TGTCCTGGCCTCATTTTTGG -3'	5'-TCCCAACGGAGTGGAGTGG-3'	129 bp
FANCD2 #1	5'-ATGGGCGAGCTTCTCTTCAC-3'	5'-AGGCAATAAGCTGGGCTCAC-3'	124 bp
FANCD2 #2	5'-TCGGTGAGTAAGTGGAGCAATG -3'	5'-AGATGAGGAAGCCAAGGTTCG-3'	122 bp
FANCD2 #3	5'-AATCCCCACAGGTACCTTTCTG -3'	5'-AACACGTGCGATACCATCTG-3'	149 bp
GDF15 #1	5'-GCATGACACATCAAGGTTGC-3'	5'-TTTTGGTTGGGGTCAAAGGC-3'	98 bp
GDF15 #2	5'-AGATCCTCCCCCTAAATACACC -3'	5'-CGGACCGGCCTTTATAGTCC-3'	136 bp
MAPK3 #1	5'-CAGCTGAGACTCAAAATGGTGTC-3'	5'-TTATTGCACCACTACACTCCAG-3'	139 bp
MAPK3 #2	5'-CTCGGAGACTGTCCTCACC -3'	5'-TGGGCTGCCGCATACATTG-3'	89 bp
NUAK1 #1	5'-AGGCCTGGTGTTCGGTTAC-3'	5'-TTCTCCTGGACTCGGAGTTG-3'	108 bp
NUAK1 #2	5'-AAGAGATGGGGCTCTAAAGGC -3'	5'-CAAACGGGTTGCTCAGCAG-3'	141 bp
NUAK1 #3	5'-GTAAGGGTCAGGCAGGAAGG -3'	5'-GTCTGGTACATTGCACCACTTG-3'	144 bp
PAK1 #1	5'-TAACAAAGCACCACCGCATC-3'	5'-ACATGTCTGGCTGTGAGATCAC-3'	89 bp
PAK1 #2	5'-ACGCCTGGGATTACGTCATC-3'	5'-AAAGGCTGCACGGAAACAAC-3'	130 bp
SENP1 #1	5'-TGGGCTTTTTCGCTTTCTCG -3'	5'-AAACTGGAACGCAAGCAAGC-3'	136 bp
SENP1 #2	5'-TTTCCATGGCAGCTTTTCGC-3'	5'-AACACGAGATAGCCTGAGATCG-3'	105 bp
TYRO3 #1	5'-TGGGCTTTTTCGCTTTCTCG -3'	5'-AAACTGGAACGCAAGCAAGC-3'	136 bp
TYRO3 #2	5'-TTTTGAGGGAAGGCTCTGGAG-3'	5'-AAACCTGAGGATCCGGCTTG-3'	114 bp
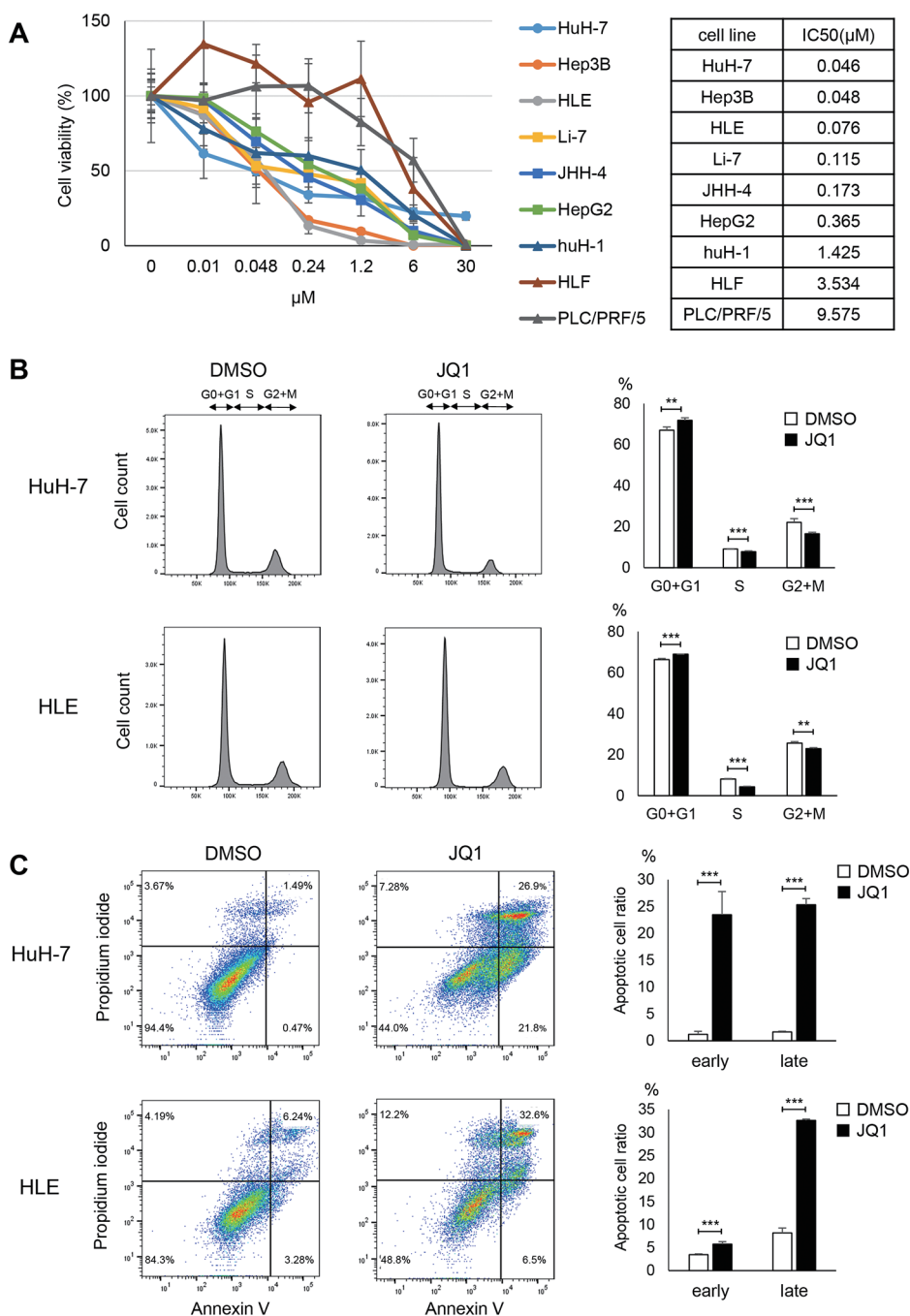
Figure 1. Anti-proliferative effects of BRD4 inhibition in HCC cells. (A) Cell viability assays using HCC cell lines treated for 5 days with the indicated concentrations of JQ1. IC50 values are shown on the right. (B,C) Results of cell cycle (B) and apoptosis (C) analyses using the indicated cell lines treated for 48 h with 1 μM JQ1. Representative results are shown on the left. Summarized results of 3 replications are shown on the right. Error bars represent SDs. **$P < 0.01$; ***$P < 0.001$.

in G0+G1 phase and decreases in S and G2+M phases in the JQ1-sensitive HuH-7, HLE and Hep3B cells (representative results in Figure 1B). In addition, Annexin V staining assays showed that JQ1 induced apoptosis in HuH-7, HLE and Hep3B cells (representative results in Figure 1C). In contrast, induction of apoptosis or cell cycle arrest by JQ1 was minimal in less sensitive huH1 and PLC/PRF/5 cells (data not shown). These results

confirm the strong anti-tumor effect of JQ1 in HCC cells, while the effects were somewhat various among cell lines.

3.2 Effects of BRD4 inhibition on gene expression profiles in HCC

We next sought to understand the mechanism underlying the anti-tumor effects of BRD4 inhibition in

HCC cells. Because previous studies showed that MYC and E2F2 are targets of BRD4 in HCC cells (11, 12), we first performed qRT-PCR analysis to assess the effect of JQ1 on their expression in HCC cells. We tested 3 HCC cell lines, HuH-7, HLE and HepG2, because these cell lines were used in earlier reports and/or they were sensitive to JQ1 (11, 12). We found that treatment with JQ1 (0.5 μM, 1.0 μM or 2.0 μM) for

6 to 24 h significantly suppressed MYC expression in all three cell lines tested. In HLE and HepG2 cells, however, MYC expression recovered within 48 h, despite continued treatment with JQ1 (Figure 2A). Expression of E2F2 was also strongly suppressed by JQ1 in all cell lines tested, and it continued to be downregulated after 48 h of JQ1 treatment (Figure 2B).
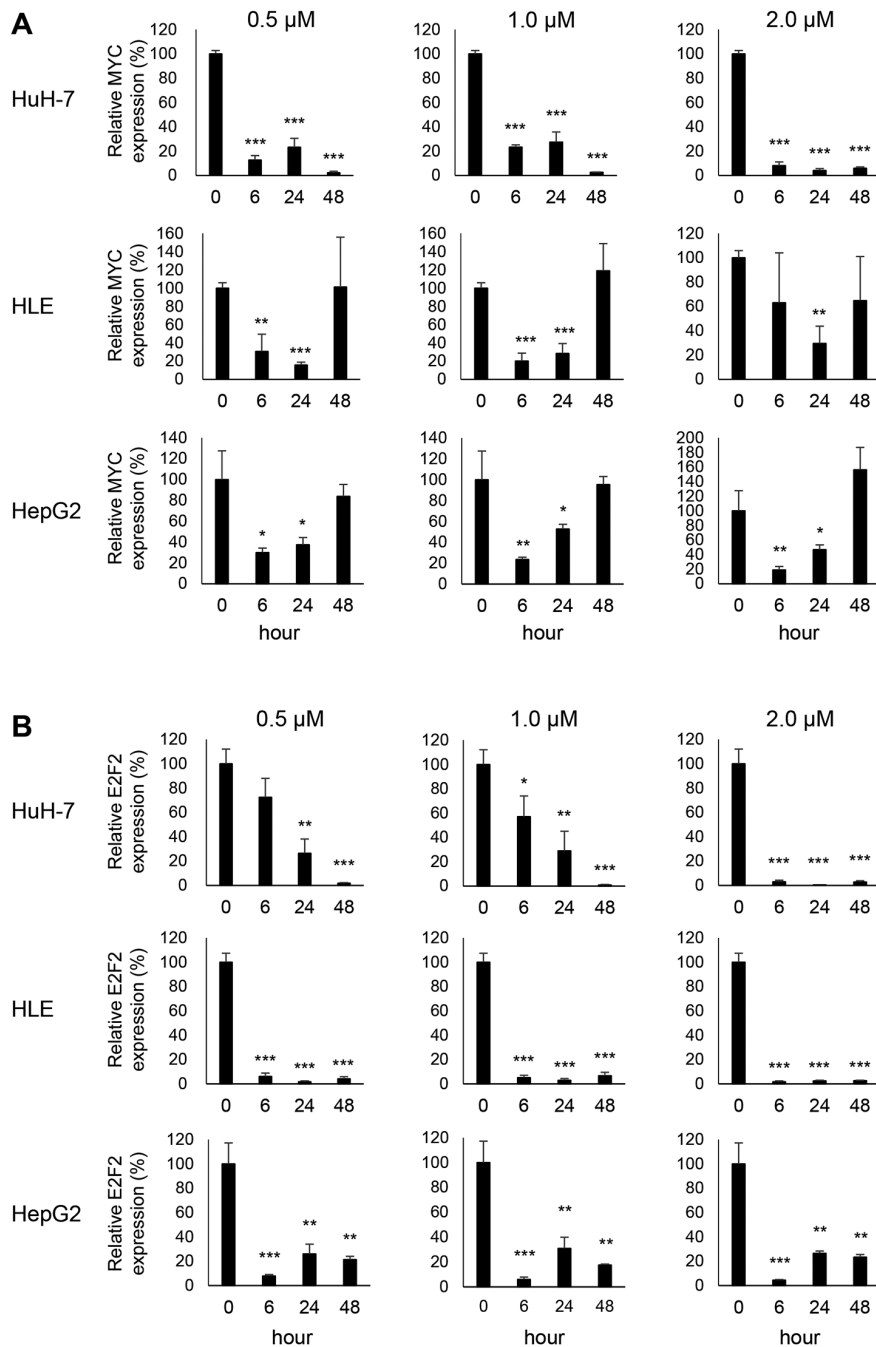
To further clarify the effects of BRD4 inhibition

Figure 2. Suppression of MYC and E2F2 by JQ1 in HCC cell lines. (A) Results of qRT-PCR analyses of MYC in the indicated HCC cell lines treated with the indicated concentrations of JQ1. (B) Results of qRT-PCR analyses of E2F2 in the indicated HCC cell lines treated with the indicated concentrations of JQ1. Results are normalized to ACTB expression. Shown are means of 3 replications; error bars represent SDs; *$P < 0.05$; **$P < 0.01$; ***$P < 0.001$.

on gene expression profiles in HCC cells, we next treated the 9 HCC cell lines with or without 1 μM JQ1 for 24 h and then performed a gene expression microarray analysis. The results showed that expression of 7112 probe sets (5497 unique genes) were significantly altered by JQ1 treatment (t-test, P < 0.05), which suggests BRD4 inhibition dramatically affects gene expression profiles in HCC cells (Figure 3A). Gene ontology analysis suggested that genes associated with membranes and intracellular organelles are significantly enriched among the downregulated genes (2304 probe sets, 1868 unique genes, Figure 3B). In addition, pathway analysis revealed that genes associated with cell cycle, apoptosis and cancer pathways were significantly enriched among the downregulated genes (Figure 3B). E2F2 was significantly downregulated by JQ1 in 8 of the 9 HCC cell lines tested (Figure 3C), but MYC was downregulated to a lesser degree than E2F2 (Figure 3C). We also
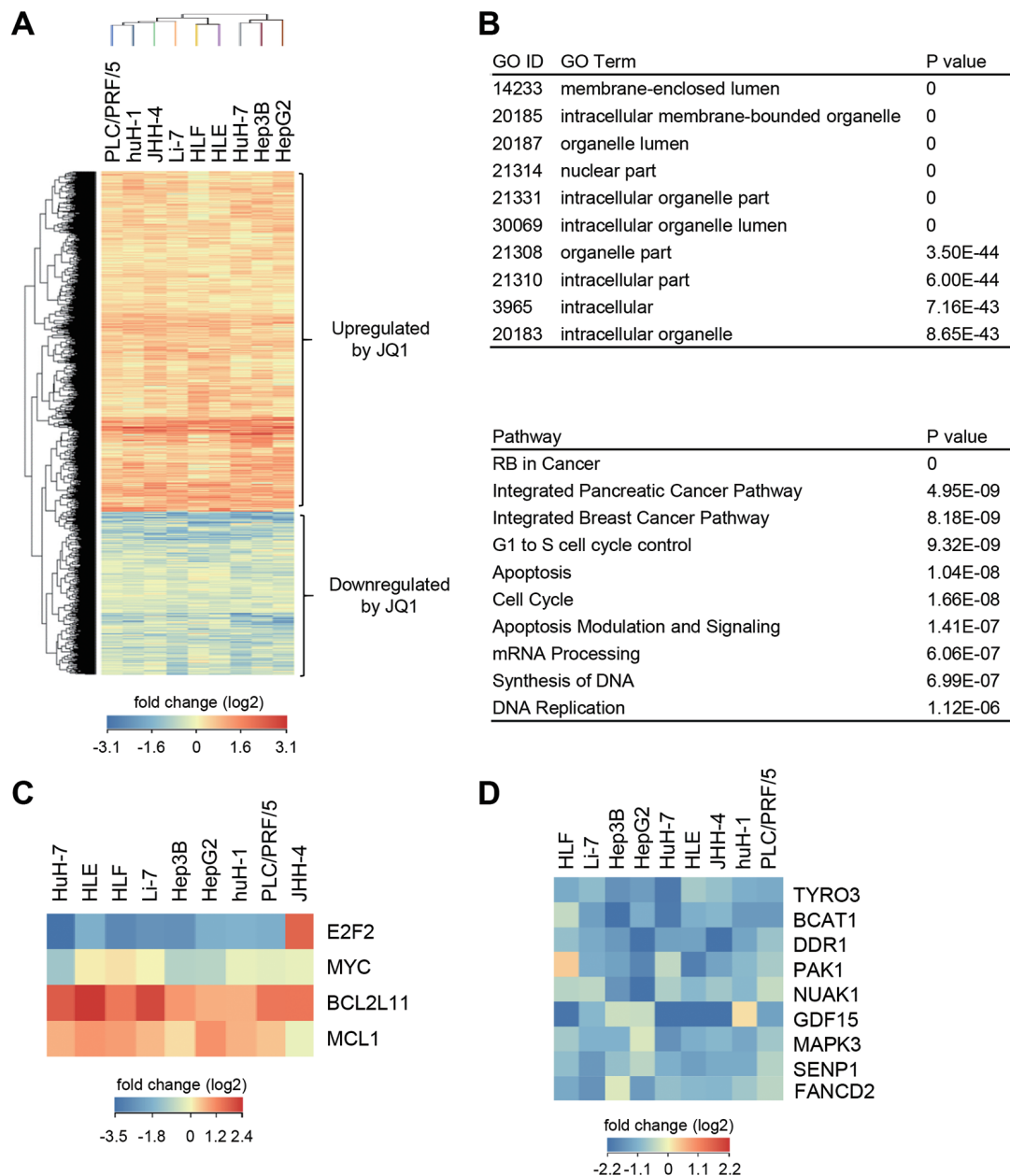

Figure 3. Effects of BRD4 inhibition on gene expression profiles in HCC cells. (A) Heat map showing expression of 7112 probe sets (5497 genes) whose expression was altered by JQ1 treatment in the indicated HCC cell lines. Results are normalized to cells treated with DMSO. (B) Results of gene ontology (GO, upper) and pathway analyses (lower) of the downregulated genes in (A). RB, retinoblastoma. (C) Heat map showing microarray results for E2F2, MYC, BCL2L11 and MCL1. (D) Heat map showing microarray results for the indicated genes.

observed that BCL2L11 and MCL1 were upregulated by JQ1 in multiple HCC cell lines, which is consistent with earlier reports (11, 16) (Figure 3C).

3.3 Identification of novel targets of BRD4 in HCC cells

To identify targets of BRD4 that could be involved in the anti-tumor effect of BRD4 inhibition, we isolated a
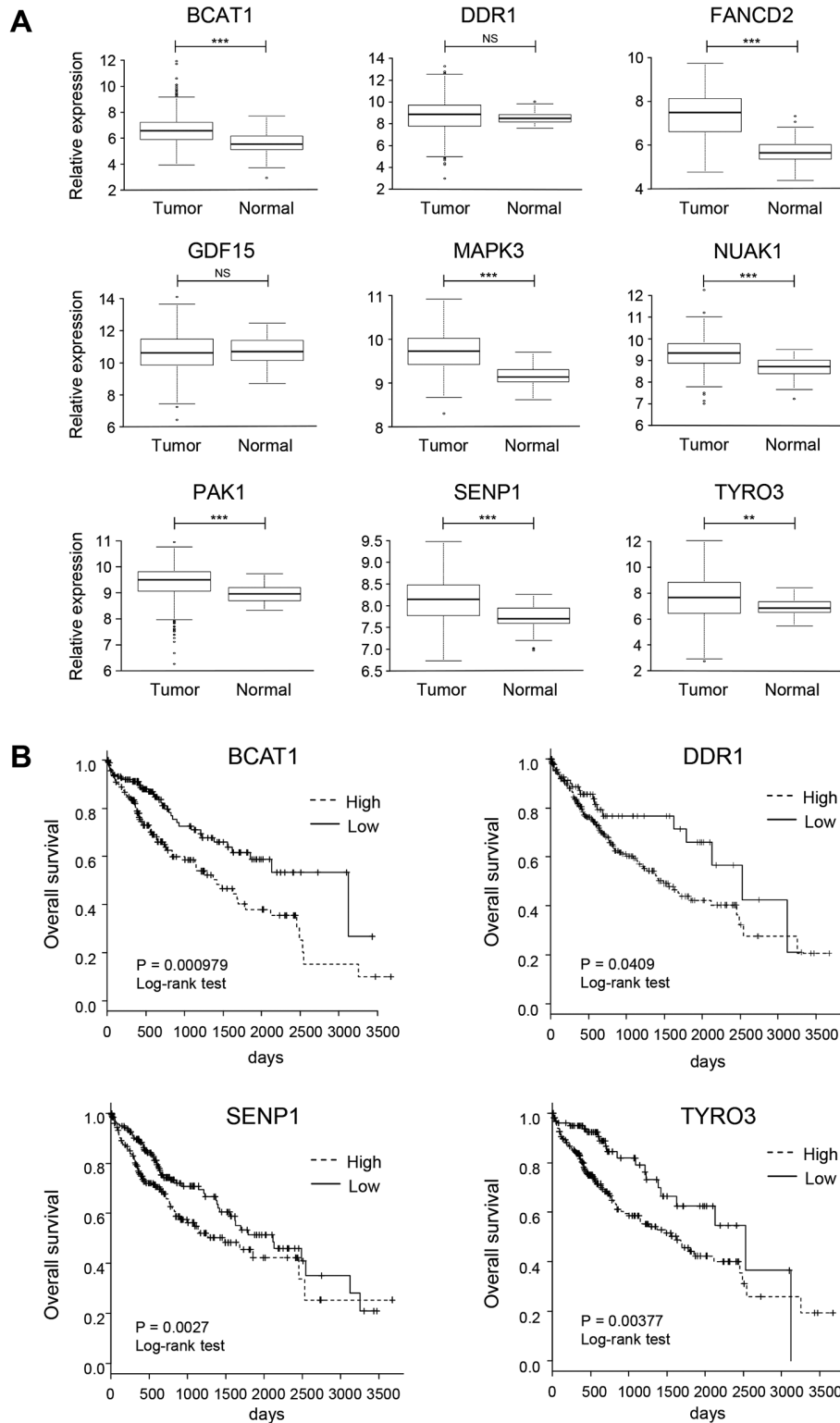

Figure 4. Expression of candidate BRD4 target genes in primary HCC tissues. (A) Summaries of the relative expression levels of the indicated genes in primary HCC tumors (n = 377) and normal liver tissues (n = 59) in the TCGA dataset. **$P < 0.01$; ***$P < 0.001$; NS, not significant. (B) Kaplan-Meier curves showing the effects of expression of the indicated genes on survival of HCC patients in the TCGA dataset (n = 365).

list of genes suppressed by JQ1 in HCC cell lines (278 probe sets, 226 unique genes) that satisfied more stringent criteria (> 2-fold, P < 0.05). Through comparison with publically available datasets for primary HCC from The Cancer Genome Atlas (TCGA), we selected for further analysis 7 genes (BCAT1, FANCD2, MAPK3, NUAK1, PAK1, SENP1 and TYRO3) whose expression levels were significantly higher in primary HCC tumors than in normal liver tissues (Figure 4A). Also selected were DDR1 and GDF15, which are reportedly associated with HCC development. Analysis of the associations between the expression levels of the selected genes and HCC patient prognosis revealed that elevated expression of BCAT1, DDR1, SENP1 and TYRO3 was associated with shorter overall survival (Figure 4B). Our microarray analysis confirmed that the selected 9 genes were significantly downregulated by JQ1 in HCC cell lines (Figure 3D).

qRT-PCR revealed that BCAT1, DDR1, FANCD2, GDF15, SENP1 and TYRO3 are upregulated in multiple HCC cell lines as compared to normal liver tissue (Figure 5). We thus selected these genes for further analysis. qRT-PCR analysis performed to validate the microarray results revealed that expression of the 6 genes was effectively suppressed by JQ1 in most of the HCC cell lines tested (Figure 6A). We next tried to confirm whether the suppressive effects on gene expression depend on direct inhibition of BRD4. To this end, we selected three HCC cell lines, HuH-7, HLE and HepG2, because these cell lines were used in earlier reports and/or they were sensitive to JQ1 (11, 12). We found that knocking down BRD4 using a specific siRNA led to decreased expression of these genes in the selected HCC cell lines, although degrees of downregulation were lesser than those by JQ1 (Figure 6B).

To determine whether these genes are direct targets of BRD4, we performed ChIP analysis using an anti-BRD4 antibody. Using the ENCODE (Encyclopedia of DNA Elements) datasets for HepG2 cells prior to that analysis, we found that levels of acetylated histone H3
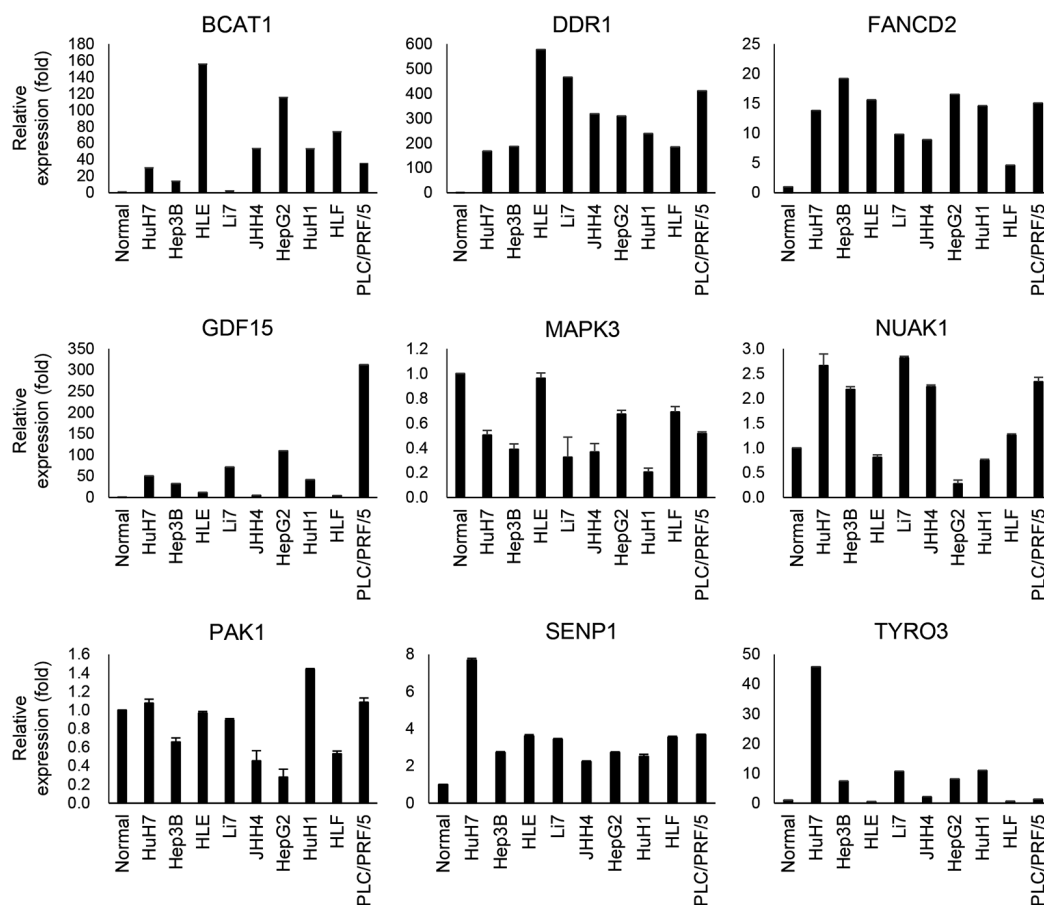

Figure 5. Results of qRT‑PCR analyses of candidate BRD4 target genes in normal liver tissue and HCC cell lines. Results are normalized to ACTB expression. Shown are means of 3 replications; error bars represent SDs.
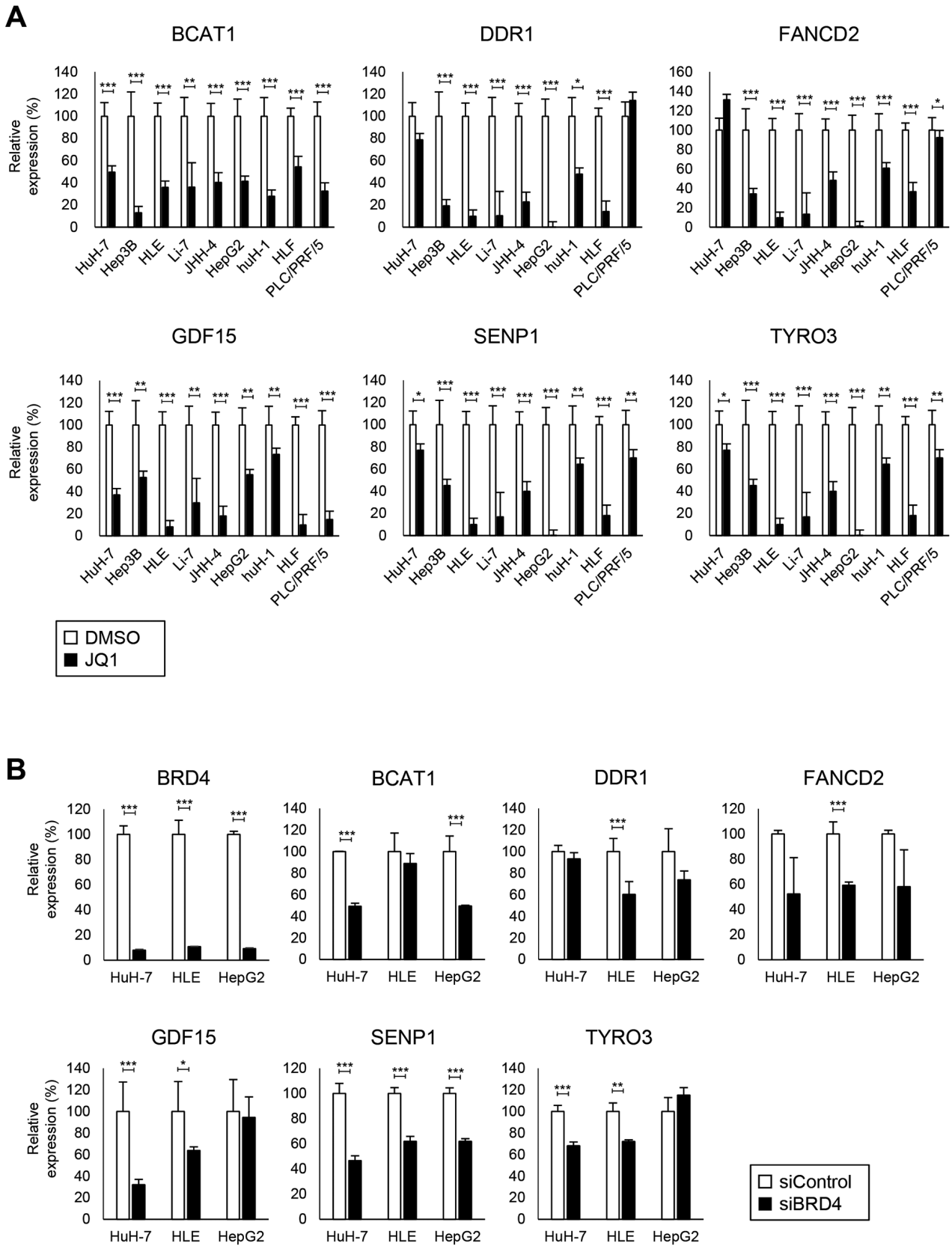
A

B

Figure 6. Inhibition of BRD4 suppresses expression of candidate BRD4 target genes in HCC cells. (A) Results of qRT-PCR analyses of the indicated genes in HCC cell lines treated for 24 h with DMSO or JQ1 (1 μM). (B) Results of qRT-PCR analyses of the indicated genes in HCC cell lines transfected with a control siRNA or a siRNA targeting BRD4. Results are normalized to ACTB expression. Shown are means of 3 replications; error bars represent SDs; *$P < 0.05$; **$P < 0.01$; ***$P < 0.001$.
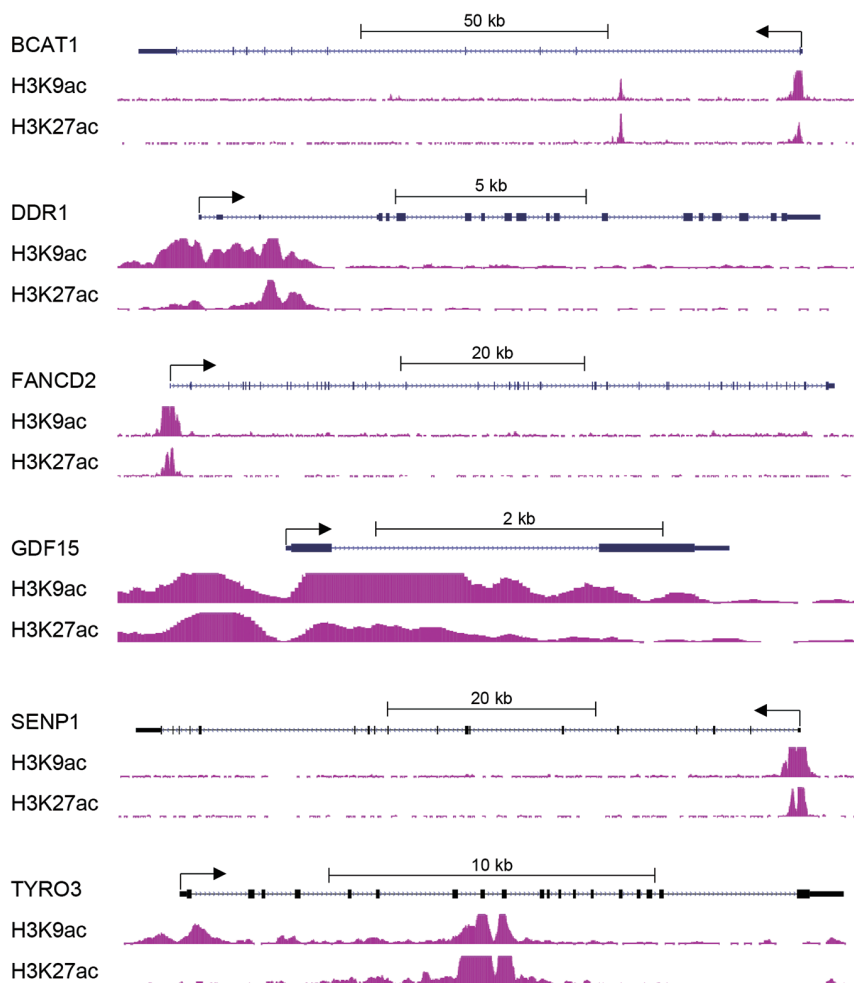
Figure 7. Histone acetylation status of candidate BRD4 target genes in HepG2 cells. ChIP-seq results of acetylated histone H3 lysine 9 (H3K9ac) and H3K27ac were obtained from the ENCODE dataset.

lysine 9 and 27 were elevated at the promoters and transcription start sites of the 6 genes (Figure 7). Further analysis of these regions using ChIP-qPCR revealed BRD4 to be significantly enriched in all the regions tested (Figure 8).

4.　Discussion

In the present study, we first confirmed the anti-tumor effects of BRD4 inhibition using a series of HCC cell lines. We found that treatment with the BRD4 inhibitor JQ1 suppressed HCC cell proliferation by inducing cell cycle arrest and apoptosis, though the sensitivities to JQ1 varied among cell lines. The mechanism underlying the anti-tumor effect of BRD4 inhibition in HCC cells was investigated in several earlier studies. For instance, Hong et al. showed that E2F2 is a direct target of BRD4 and that the BRD4-E2F2 cell cycle circuit is an important therapeutic target in HCC[12]. We found that JQ1 strongly suppressed E2F2 expression in most of the HCC cell lines tested.

MYC is the most well-studied target of BRD4 in various types of cancer, though the effect of BRD4 inhibition on MYC in HCC remains controversial. Li et al. showed in multiple HCC cell lines that JQ1 suppresses MYC in a dose and time dependent manner (11). However, Hong et al. observed no significant effect of JQ1 on MYC expression in HCC cell lines[12]. Our qRT-PCR analysis revealed that treatment with JQ1 rapidly (6 to 24 h) suppressed MYC expression in multiple HCC cell lines, but MYC expression then recovered with more prolonged treatment. Consistent with the qRT-PCR results, our microarray analysis also showed that treatment with 1 μM JQ1 for 24 h only moderately suppressed MYC in HCC cell lines. These data may explain the earlier contradictory results for JQ1 effects on MYC expression. We also observed that JQ1 treatment led to upregulation of genes reportedly associated with the drug's effects. BCL2L11 (BIM) is a pro-apoptotic gene and its upregulation
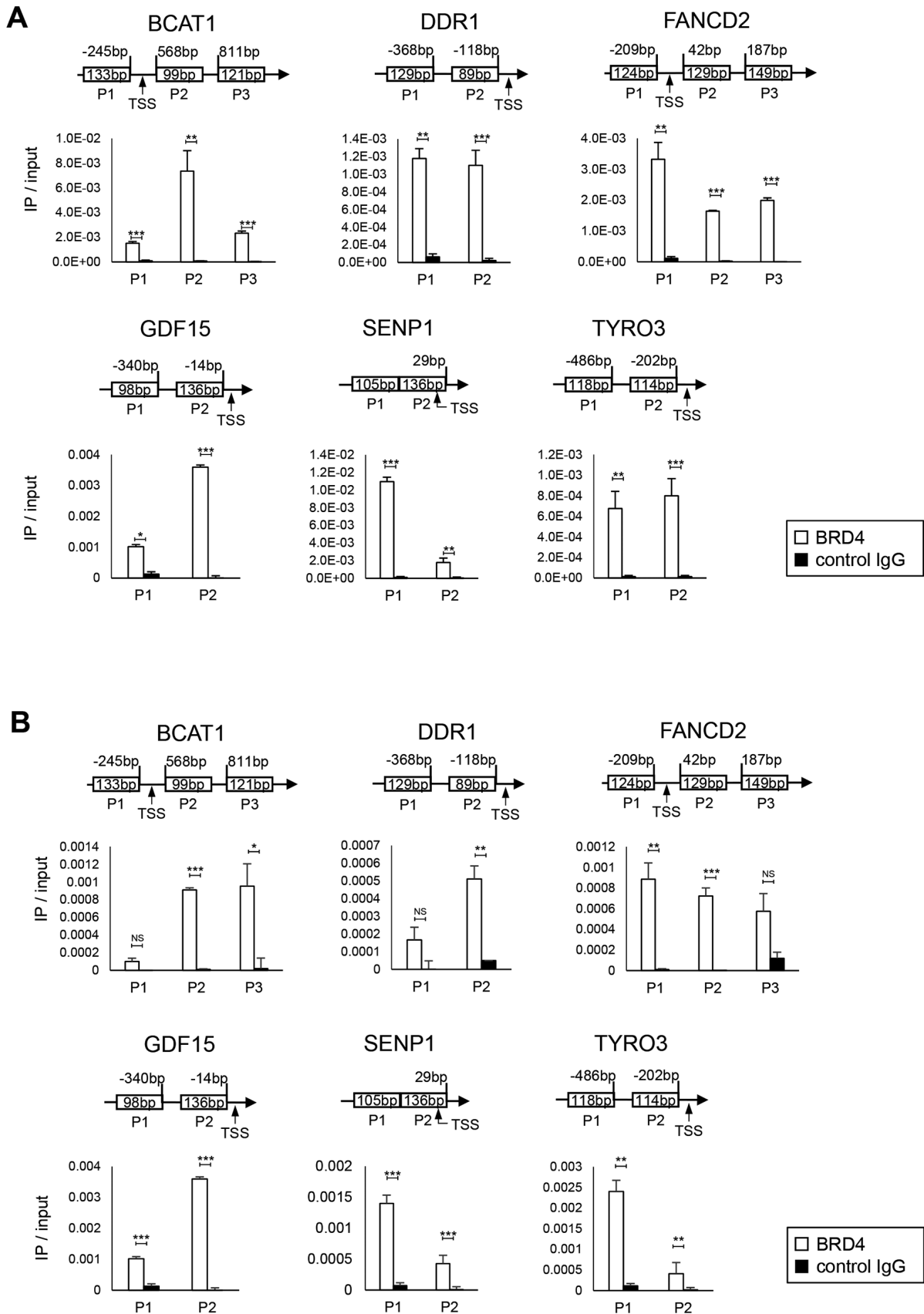
Figure 8. BRD4 binding to candidate target genes in HCC cells. (A,B) Summarized results of ChIP-qPCR analyses of the indicated genes in HepG2 (A) and HuH-7 cells (B). Regions analyzed are shown at the top, and locations relative to the transcription start site (TSS) are also indicated. Results are normalized to respective input DNAs. Shown are means of 3 replications; error bars represent SDs; *$P < 0.05$; **$P < 0.01$; ***$P < 0.001$.

by JQ1 triggers apoptosis in HCC cells (11). MCL1 is also reportedly induced by JQ1 in HCC cells, though it antagonizes the JQ1-triggered anti-tumor effect[16].

To identify novel targets of BRD4 that could be associated with the anti-tumor effects of BRD4 inhibition in HCC cells, we performing a gene expression microarray analysis, which revealed that a number of genes with oncogenic properties are downregulated by JQ1 treatment. Branched-chain amino acid transaminase 1 (BCAT1, also known as ECA39) encodes the cytosolic form of the enzyme, which catabolizes branched-chain amino acids (BCAAs)[17]. Expression of BCAT1 is increased in HCC tissues, where it promotes cell proliferation, migration and invasion[18, 19]. One recent study showed that overexpression of BCAT1 in HCC may also induce circulating tumor cell release by triggering epithelial-mesenchymal transition, and that BCAT1 may be an important marker of HCC metastasis[20].

Discoidin domain receptor 1 (DDR1) belongs to a receptor tyrosine kinase family characterized by a 155-amino acid discoidin homology domain in its extracellular region[21]. In HCC, elevated DDR1 expression is associated with early recurrence and promotion of HCC cell migration and invasion via activation of matrix metalloproteinase-2 (MMP-2) and MMP-9[22, 23]. Another study showed that miR-199a-5p targets DDR1, and that downregulation of miR-199a-5p is one of the mechanisms underlying DDR1 overexpression in HCC[24].

Growth differentiation factor 15 (GDF15), also known as macrophage inhibitory cytokine-1 (MIC-1), is a member of the transforming growth factor-β (TGF-β) superfamily and is overexpressed in multiple tumor types[25]. GDF15 is induced in hepatocytes by hepatitis C virus infection, and serum GDF15 levels are elevated in patients with liver cirrhosis or HCC[26, 27]. Although genetic ablation of Gdf15 does not affect HCC development in mice, recent studies showed that GDF15 promotes proliferation, migration, invasion and metastasis by HCC cells[28-30]. Moreover, GDF15 secreted by HCC cells enhances tumor angiogenesis[30, 31]. These results suggest that GDF15 is involved in the pathogenesis of HCC and could be a diagnostic marker and a therapeutic target.

Fanconi anemia complementation group D2 (FANCD2) is a member of Fanconi anemia (FA) protein family. FA is a rare genetic disorder characterized by congenital abnormalities, progressive pancytopenia and increased cancer susceptibility[32]. Komatsu et al. reported that high FANCD2 expression is associated with poorer prognosis in HCC patients and that FANCD2 knockdown diminished the proliferation and invasiveness of HCC cells[33].

SUMOylation, which entails conjugation of small ubiquitin-like modifier (SUMO) proteins to target substrates, is an important post-translational protein modification[34]. Specific SUMO protease 1 (SENP1) is essential for stabilization of hypoxia-inducible factor 1α (HIF-1α) through de-conjugation of SUMO proteins (deSUMOylation)[35]. A recent study showed that hypoxia enhances the stemness of HCC cells by promoting HIF-1α deSUMOylation by SENP1, and that SENP1 is a direct target of HIF-1/2α[36]. This suggests the existence of a positive feedback loop between SENP1 and HIF-1α. SENP1 also reportedly regulates hepatocyte growth factor (HGF)-induced migration and invasion by HCC cells[37].

TYRO3 belongs to the TAM (TYRO3, AXL and MERTK) family of receptor tyrosine kinases[38]. Duan et al. recently reported that TYRO3 is frequently overexpressed in primary HCC, and that knocking down TYRP3 suppresses proliferation, ERK phosphorylation and cyclin D1 expression in HCC cells[39]. TYRO3 is also reportedly a target of miR-7, which functions as a tumor suppressor in HCC[40]. TYRO3 promotes HCC cell proliferation, migration and invasion through activation of the PI3K/AKT pathway, and its expression in HCC cells is markedly increased in cells exhibiting sorafenib resistance. Induction of miR-7 effectively overcomes sorafenib resistance by suppressing TYRO3, which suggests the miR-7/TYRO3 axis could be a useful therapeutic target in sorafenib-resistant HCC[40].

Using ChIP-qPCR analysis, we confirmed BRD4 binding within the promoter and transcription start site regions of the genes we identified in this study. Taken together with the earlier reports summarized above, our results suggest that these genes are direct targets of BRD4 and that their suppression may contribute to the anti-tumor effect of BRD4 inhibition in HCC cells. However, there are several limitations to our study. It is well documented that the anti-tumor mechanism of BRD4 inhibition is attributable to the removal of BRD4 from super-enhancers, which regulate expression of key oncogenes, including MYC[6]. It is therefore possible that the candidate BRD4 target genes are regulated by enhancers located at genomic regions distant from the transcription start sites. Identification of such enhancer regions is challenging, and further genomic and epigenomic analyses to identify distant regulatory regions will be necessary to address this issue. Second,

although the HCC cell lines tested in this study exhibit different sensitivities to JQ1, we could not identify genes associated with JQ1 sensitivity. The candidate BRD4 target genes we identified were suppressed by JQ1 in most of the HCC cell lines tested, irrespective of their sensitivity to JQ1. Third, we found that knockdown of BRD4 was less effective to suppress candidate BRD4 target genes than JQ1, suggesting that the effect of JQ1 on these genes may be attributable to inhibition of other targets than BRD4.

In summary, we show that BRD4 inhibition significantly affects gene expression profiles in HCC cells. Among the genes downregulated by BRD4 inhibition, we identified a series of novel BRD4 target genes. Our findings suggest that suppression of multiple genes with oncogenic function may contribute to the anti-tumor effect of BRD4 inhibition in HCC.

5. Acknowledgements

6. Conflict of interests

All authors declare no conflict of interest.

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肝細胞癌における BRD4 阻害の抗腫瘍効果メカニズムの解析

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　BRD4 を含む BET ファミリー蛋白質はアセチル化ヒストンのリーダー蛋白質であり，転写調節において重要な役割を担っている．BRD4 阻害剤は様々な悪性腫瘍において抗腫瘍効果を示す事が報告されている．今回我々は，肝細胞癌における BRD4 阻害の抗腫瘍効果メカニズムの解明を目的とした．まず BRD4 阻害剤 JQ1 の抗腫瘍効果を 9 種類の肝癌細胞株を用いて検証した結果，増殖抑制，細胞周期停止，アポトーシスの誘導が認められた．次に我々は，JQ1 が遺伝子発現に与える影響をマイクロアレイによって解析した．その結果，JQ1 が細胞周期やアポトーシス関連遺伝子に強い影響を与えること，そして BCAT1，DDR1，GDF15，FANCD2，SENP1，TYRO3 など複数の肝細胞癌関連遺伝子の発現を抑制することを見いだした．これらの遺伝子のプロモーター領域への BRD4 結合が確認されたことから，これらは肝細胞癌における BRD4 の標的遺伝子と考えられた．今回の結果から，BRD4 阻害剤は複数の癌関連遺伝子を抑制することで，肝細胞癌に対する抗腫瘍効果を示すと考えられた．