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Original Article

Dysregulation and activities of ubiquitin specific peptidase 2b in the pathogenesis of hepatocellular carcinoma

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Abstract: Ubiquitin specific peptidase-2 (USP2) plays important roles in a myriad of cellular activities through deubiquitinating target proteins and its implications in various diseases, especially cancers, are starting to emerge. Our current understanding on USP2 expression in subjects with hepatocellular carcinoma (HCC) and its roles in the pathogenesis of HCC is limited. In this study, we found that USP2 protein and mRNA levels were significantly dysregulated in HCC tumor (HCC-T) when compared to adjacent non-tumor (HCC-NT) or normal liver tissues from both human and mouse HCC model. Among the USP2 isoforms, USP2b was the predominant isoform in the normal liver and markedly down-regulated in HCC-T tissues in both human and mice. Data from overexpression, chemical inhibition and knockout studies consistently demonstrated that USP2b promoted cell proliferation, colony formation and wound healing in HepG2 and Huh 7 cells. On the other hand, USP2b exhibited proapoptotic and pronecrtotic activities through enhancing bile acid-induced apoptosis and necrosis in both HepG2 and Huh 7 cells. Unbiased proteomic analysis of USP2-knockout (KO) and parental HepG2 cells resulted in identification of USP2-regulated downstream target proteins involved in cell proliferation, apoptosis, and tumorigenesis, including serine/threonine kinase 4 (STK4), epidermal growth factor receptor (EGFR), dipeptidyl peptidase 4 (DPP4) and fatty acid binding protein 1 (FABP1). In conclusion, USP2b expression was dysregulated in subjects with HCC and contributed to the pathogenesis of HCC by promoting cell proliferation and exerting proapoptotic and pronecrotic activities. The findings provide the molecular basis for developing therapies for HCC through modulating USP2b expression or activities.

Keywords: HCC pathogenesis, USP2b dysregulation, apoptosis and necrosis, cell proliferation, ubiquitination and deubiquitination

Introduction

Hepatocellular carcinoma (HCC) is the sixth most common cancer worldwide and the third leading cause of cancer-related deaths [1]. HCC accounts for more than 90% of primary liver cancers with incidence rates highest in Asia and Africa and is increasing at a worrisome rate in western countries including United States [1, 2]. At present, there are limited options in treating HCC patients [3]. There is urgent need to develop effective therapies for HCC. However, challenges remain mainly due to the complexity of HCC pathogenesis and lack of fully understanding the underlying molecular mechanisms.

The ubiquitin-proteasome system is the major pathway for selective protein degradation in eukaryotic cells [4, 5]. Ubiquitination can be reversed by deubiquitinating enzymes (DUBs) [6, 7]. As a member of DUBs, ubiquitin specific peptidase 2 (USP2) removes ubiquitin from ubiquitinated target proteins, thus preventing proteasomal degradation of the target proteins [8-11]. USP2 has been reported to play critical roles in many physiological and pathological activities including tumorigenesis, circadian clock, metabolism, inflammation, and ion channel regulation [12]. Three isoforms of USP2 have been reported including USP2a (USP2-69), USP2b (USP2-45) and USP2c (USP2-41)

[13-15] with USP2a being most studied among the isoforms. Currently, it remains unclear or controversial which isoforms are expressed in the liver [14, 16, 17]. Furthermore, our understanding of USP2 and its isoforms in the pathogenesis of HCC, especially USP2b, is limited [18, 19].

In this study, we found that both USP2 protein and mRNA levels were significantly decreased in HCC tumor (HCC-T) compared with adjacent non-tumor (HCC-NT) or normal liver tissues from both human and mice. Among the USP2 isoforms, USP2b was the predominant isoform in the liver and markedly down-regulated in HCC-T. USP2b exhibited tumorigenic activities through promoting cell proliferation, colony formation and wound healing. On the other hand, USP2b significantly enhanced bile acid-induced apoptosis and necrosis. Proteomic profiling of parental HepG2 and USP2 knockout (USP2-KO) cells resulted in identification of USP2-regulated downstream target proteins involving in cell proliferation, apoptosis, and tumorigenesis. The findings revealed the novel activities of USP2b and its contribution to the pathogenesis of HCC.

Materials and methods

Cells and cell culture

Human hepatoma cell line HepG2 (cat#: HB-8065) and Huh 7 (cat#: PTA-4583) cells were obtained from American Type Culture Collection (ATCC). USP2 knockout (USP2-KO) cells were generated using parental HepG2 cells and pLentiCRISPR v2 USP2 sgRNA (5'-GGTCCCGCATGTAGAGCCTC-3') construct (GenScript). USP2 protein was knocked out with an insertion in both cell clones. The sgRNA was targeted to the core region of USP2 so all the isoforms of USP2 were knocked out. Two independent USP2-KO cell lines (3A and 3E) with similar cell morphology and growth characteristics were selected for the study.

Human liver samples

Human liver tissue microarray slides including a total of 76 normal, 38 HCC-NT and 115 HCC-T samples with 10 pairs of HCC-NT and HCC-T samples were purchased from US Biomax (Cat# BC03119b-SC2, LV241a-D024, LVN241-T202 and LVN801-H139). Twelve normal human liver and 21 HCC-T with 8 paired HCC-NT tissues were obtained through the Cooperative Human Tissue Network. The study was approved by the Institutional Review Board (IRB) at the University of Rhode Island (URI).

Mouse HCC model and liver tissue samples: Farnesoid X receptor (FXR) is a master regulator of bile acid homeostasis and FXR knockout mice (FXR-KO) spontaneously develop HCC as they age (13 to 15 months) [20, 21]. FXR-KO (stock no: 007214) and C57BL/6J wt (stock no: 000664) were obtained from the Jackson Laboratory (Bar Harbor, Maine). Mice were fed a regular chow diet. A total of 26 FXR-KO and 16 C57BL/6J wt mice at the ages of 14-18 months were used for the study. All 26 FXR-KO mice had liver tumor formation. Paired HCC-T and HCC-NT tissue sections were collected. Liver samples were snap frozen in liquid nitrogen when the mice were euthanized. The study was approved by the Institutional Animal Care and Use Committee (IACUC) of URI.

Expression plasmid construct of USP2b

The expression construct for human USP2b were custom-made and expressed in the pcDNA3.1(+) vector. Codons were optimized to enhance the expression in human cell lines. The expression of USP2b construct was confirmed by transfection into HepG2 and Huh 7 cells, followed by western blot detection.

Cell proliferation assays

HepG2, Huh 7 and USP2-KO cells were transfected with USP2b or pcDNA vector with or without treatment of USP2 specific inhibitor ML364 (MedChem Express) [22]. Cell viability was detected by the MTS assays (Promega). Cell proliferation assays were also carried out by viable cell counting.

Colony formation assays

HepG2 and Huh 7 cells were transfected with USP2b or pcDNA vector, followed by growth for 7 days. Colonies were quantified using ImageJ cell counter plug in.

Wound-healing assays

HepG2 and Huh 7 cells were seeded in 6-well plates in serum starved media and transfected

with USP2b or pcDNA vector. Cells were counted and seeded in triplicate into CytoSelect 24-Well Wound Healing Assay plates (Cell Biolabs Inc). Photographs were taken at time 0 when wound was created, as well as 24 or 48 h post wound formation. Average percent wound closure was calculated using ImageJ based upon 3 random fields per well.

Apoptosis and necrosis assays

HepG2 and Huh 7 cells were transfected with USP2b or pcDNA vector, followed by treatment with various concentrations of chenodeoxycholic acid (CDCA) (100, 200, 300, 400 and 500 μM) or vehicle for various time points (0, 2, 4, 8, 12, 24 and 48 h). Apoptotic and necrotic activities were detected with the RealTime-Glo Annexin V Apoptosis and Necrosis Assay (Promega).

Quantitative real-time PCR

Total RNA isolation from human or mouse liver tissues, cDNA synthesis and TaqMan real-time PCR assays were performed as previously described [23]. TaqMan probes including human total USP2 (Hs00275859_m1), USP2b (Hs01592505_m1), mouse Usp2b (Mm01168-648) were purchased from Thermo Fisher Scientific.

Immunohistochemistry (IHC)

Liver tissue slides were blocked with 10% normal donkey serum (Abcam) followed by incubation with goat anti-mouse USP2 IgG (AF5804, R & D Systems) or pre-immunized serum (Biomatik). The slides were then incubated with goat IgG VisUCyte HRP Polymer (VC004, R & D systems). ImageJ was used to quantify USP2 staining. The specificity of the goat anti-USP2 antibodies were confirmed by Western blot with human liver tissues (data not shown).

Western blot

Lysates from HepG2 wt and USP2-KO cells or human liver tissues were used for Western blot as previously described [23]. Antibodies against human FABP1 (sc-271591), STK4 (sc-515051), EGFR (sc-373746), GAPDH (sc-365062) and goat-anti-mouse IgG-HRP (sc-2005) were purchased from Santa Cruz Biotechnology. Antibody against human DPP4 (AF1180-SP) was obtained from R&D Systems. Rabbit anti-goat IgG (H+L) HRP (81-1620) was from Thermo Fisher Scientific.

Proteome analysis

The parental HepG2 wt and two independent USP2 knockout cells, USP2-KO (3A) and USP2-KO (3E) were used with three replicates for each type of cells. The cell pellets were subjected for protein extraction followed by trypsin digestion. Equal amounts (1 μg/μL) of desalted tryptic peptides were analyzed by LC-MS/MS analysis. The proteomic platform was followed as described previously [24]. Peptide spectrum matching of MS/MS spectra of each file was searched against UniProt protein database using the Sequest algorithm within Proteome Discoverer v 2.3 software (Thermo Fisher Scientific, San Jose, CA). The relative label-free quantitative and comparative among the samples were performed using the Minora algorithm and the adjoining bioinformatics tools of the Proteome Discoverer 2.3 software. Every protein/peptide identified with 1% false discovery rate (FDR) confidence level for the sequence assignments was included in the analysis. Kyoto encyclopedia of genes and genomes (KEGG) pathways and ShinyGo gene ontology enrichment analysis were performed. All proteins/peptides are normalized and the ratio, *p*-value and the abundance of each protein in group and/or sample were determined.

Statistical analysis

Student t-test was applied to pair-wise comparison for normally distributed data. One-way ANOVA was applied to analyze data with multiple groups, followed by Tukey post-hoc test for multiple comparisons. A *p*-value of 0.05 or lower was considered statistically significant.

Results

USP2 expression was dysregulated in patients with HCC

USP2 plays important roles in a myriad of cellular activities and its implications in various diseases are starting to be recognized recently. To determine whether USP2 expression was altered in HCC, the expression levels of USP2 protein and mRNA in normal, HCC-NT and HCC-T liver tissues were evaluated by IHC and real-time PCR. As shown in **Figure 1A** and **1B**, USP2 protein levels were significantly reduced

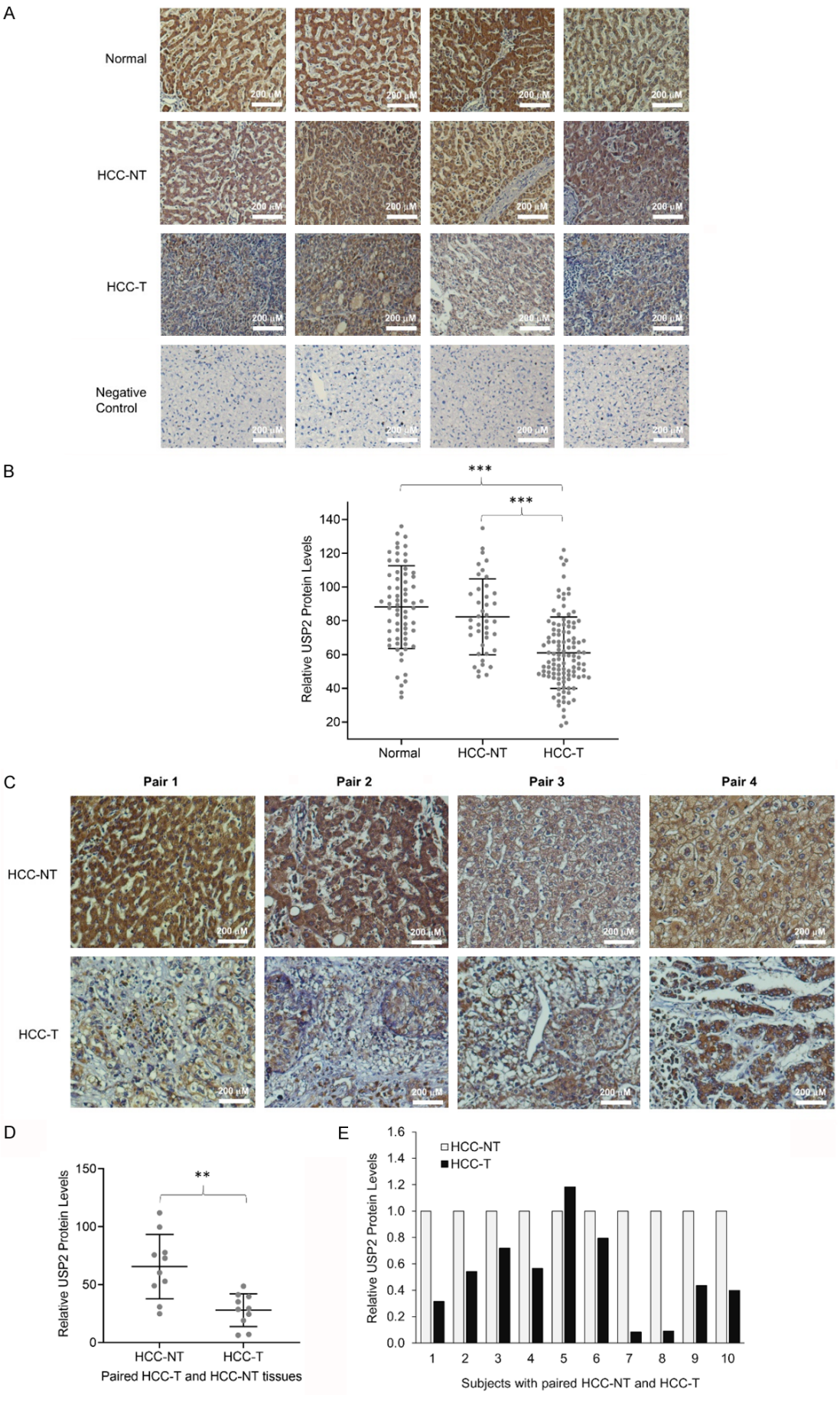

A
Normal
HCC-NT
HCC-T
Negative Control
200 μM
B
***
***
Relative USP2 Protein Levels
140
120
100
80
60
40
20
Normal
HCC-NT
HCC-T
C
Pair 1
Pair 2
Pair 3
Pair 4
HCC-NT
HCC-T
200 μM
D
**
Relative USP2 Protein Levels
150
100
50
0
HCC-NT
HCC-T
Paired HCC-T and HCC-NT tissues
E
HCC-NT
HCC-T
Relative USP2 Protein Levels
1.6
1.4
1.2
1.0
0.8
0.6
0.4
0.2
0.0
1
2
3
4
5
6
7
8
9
10
Subjects with paired HCC-NT and HCC-T

Figure 1. USP2 protein expression levels were reduced in HCC-T tissues from HCC patients. (A) Representative total USP2 protein staining with goat anti-human USP2 IgG in the tissues of human normal liver (n=76), HCC-NT (n=38) and HCC-T (n=115) by IHC. As negative controls, human normal liver tissues (n=6) were also stained with goat pre-immune serum. (B) Quantification of the USP2 protein levels were performed with ImageJ. (C) Representative USP2 protein staining in four paired HCC-T and HCC-NT tissues (n=10) by IHC. (D) USP2 protein levels in ten paired HCC-NT and HCC-T tissues as groups as well as (E) individual pairs. **P<0.01 and ***P<0.001 in students' t-test for pair-wise comparison or one-way ANOVA, followed by Tukey post-hoc test for multiple comparisons.

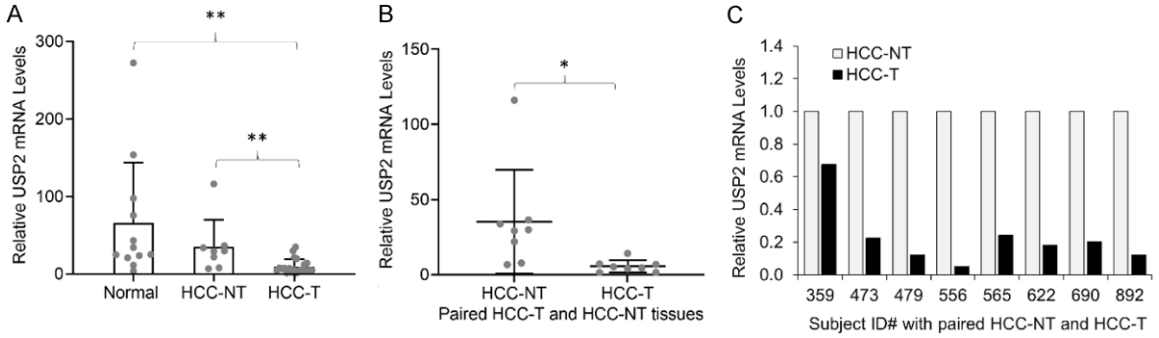


Figure 2. USP2 mRNA expression levels were decreased in HCC-T tissues from HCC patients. (A) The expression levels of USP2 mRNA in human normal liver (n=12), HCC-NT (n=8) and HCC-T (n=21) detected by real-time PCR. (B) The expression levels of USP2 mRNA in paired HCC-NT and HCC-T tissues as groups as well as (C) individual pairs (n=8). *P<0.05 and **P<0.01 in students' t-test for pair-wise comparison or one-way ANOVA, followed by Tukey post-hoc test for multiple comparisons.

in HCC-T (n=115) compared to normal liver (n=76) (P<0.001) or HCC-NT (n=38) (P< 0.0001). USP2 protein levels were decreased in HCC-NT compared to normal liver samples but the difference did not reach a statistical significance (P=0.15). Further analysis of the 10 paired HCC-T and HCC-NT samples showed that HCC-T tissues had significantly reduced USP2 protein expression compared to the corresponding HCC-NT tissues as groups (**Figure 1C** and **1D**) (P<0.01). Among the 10 paired HCC-T and HCC-NT samples, USP2 protein expressions were reduced in HCC-T tissues in 9 pairs of HCC-T and HCC-NT samples (**Figure 1E**).

Consistent with the USP2 protein levels, USP2 mRNA levels were significantly reduced in HCC-T tissues (n=21) when compared to normal (n=12) (P<0.01) and HCC-NT tissues (n=8) (P<0.01) (**Figure 2A**). Compared HCC-NT and normal liver tissues, USP2 expression was reduced in HCC-T without reaching a statistical significance. Detailed analysis of the 8 paired HCC-T and HCC-NT tissues revealed that USP2 mRNA expression levels were significantly reduced in the HCC-T tissues as groups (P< 0.05) (**Figure 2B**) as well as individual pairs (**Figure 2C**).

USP2 expression was dysregulated in mice with HCC

FXR-knockout (FXR-KO) mice spontaneously develop HCC as they age [20, 21] and the hepatocarcinogenesis in FXR-KO mice mimics human HCC progression [25]. To determine whether Usp2 is dysregulated in HCC mouse model, the expression levels of Usp2 in HCC-T and HCC-NT tissues of FXR-KO mice and normal liver tissues from age-matched C57BL/6J wt mice were evaluated. Consistent with the findings from HCC patients, Usp2 mRNA expression levels were markedly decreased in HCC-T when compared to HCC-NT and normal liver tissues (**Figure 3A**). It was also noted that Usp2 expression levels were significantly reduced in HCC-NT when compared to normal liver tissues (**Figure 3A**). Thus, Usp2 expression in HCC-NT represented the intermediate status between normal and HCC-T tissues. Detailed analysis of the individual pairs of HCC-T and HCC-NT revealed that Usp2 expression levels were reduced in 87% of the pairs (**Figure 3B**).

USP2b is the predominant isoform of USP2 in the liver

Three functional isoforms of USP2 have been reported, including USP2a (USP2-69), USP2b

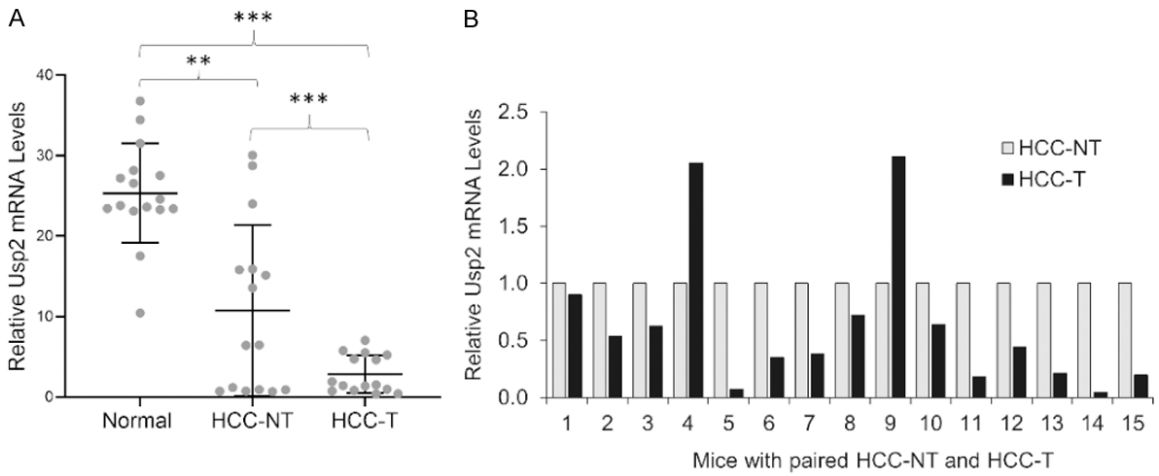


Figure 3. Dysregulation of Usp2 mRNA expression in HCC-T tissues from HCC mice. A. The expression levels of Usp2 mRNA in mouse normal liver (n=16), HCC-NT (n=15) and HCC-T (n=15) samples. B. The expression levels of USP2 mRNA in paired HCC-T and HCC-NT tissues. **P<0.01 and ***P<0.001 in students' t-test for pair-wise comparison or one-way ANOVA, followed by Tukey post-hoc test for multiple comparisons.

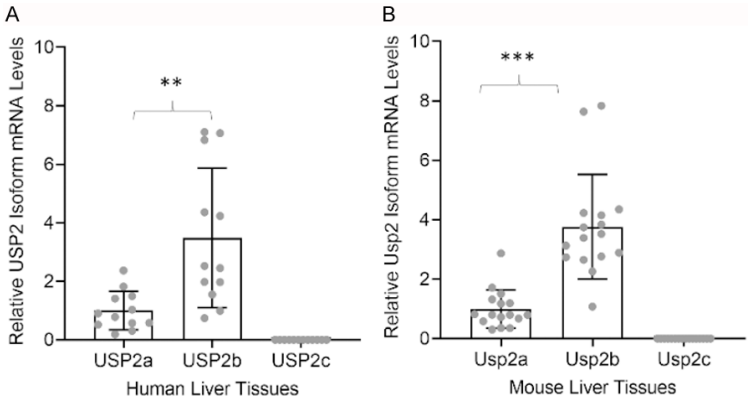


Figure 4. USP2b is the predominant USP2 isoform in the liver. (A) The relative mRNA expression levels of USP2a, USP2b and USP2c in normal liver tissues from human (n=12) and (B) mice (n=16). **P<0.01 and ***P<0.001 in one-way ANOVA, followed by Tukey post-hoc test for multiple comparisons.

(USP2-45) and USP2c (USP2-41) [13-15]. It remains unclear which USP2 isoforms are expressed in the liver [14, 16, 17]. In this study, isoform-specific probes were used to establish USP2 isoform-specific expression profiles in both human and mouse normal liver tissues. As shown in **Figure 4A** and **4B**, both isoforms USP2a and USP2b were readily detected while USP2c was under detection by real-time PCR in both human (n=12) and mouse liver (n=16). Of the two detectable isoforms, USP2b mRNA levels were approximately 3.5-fold more abundant than USP2a in both human and mouse liver (P<0.01 or P<0.001). Therefore, USP2b is the predominant isoform in the liver and our subsequent studies were focused on USP2b.

USP2b was significantly downregulated in HCC-T tissues from both human and mice

As shown in **Figure 5A**, the expression levels of USP2b mRNA were significantly reduced in HCC-T (n=18) compared to HCC-NT (n=8) and normal tissues (n=12) in human (P< 0.001). Consistent with the mRNA levels, USP2b protein levels were significantly decreased in HCC-T tissues (n=8) when compared to the normal (n=8) and HCC-NT tissues (n=8) (**Figure 5B** and **5C**). Further analysis with eight individual pairs of HCC-T and HCC-NT tissues revealed that USP2b was markedly downregulated in HCC-T tissues as groups as well as individual pairs when compared to HCC-NT tissues (**Figure 5D** and **5E**). The expression profiles of USP2b thus resembled that of the total USP2 expression (**Figures 1** and **2**) in the HCC subjects, consistent with the finding that USP2b is the predominant isoform of USP2 in the liver (**Figure 4**).

Consistent with the results from HCC patients, Usp2b mRNA expression in HCC mouse model

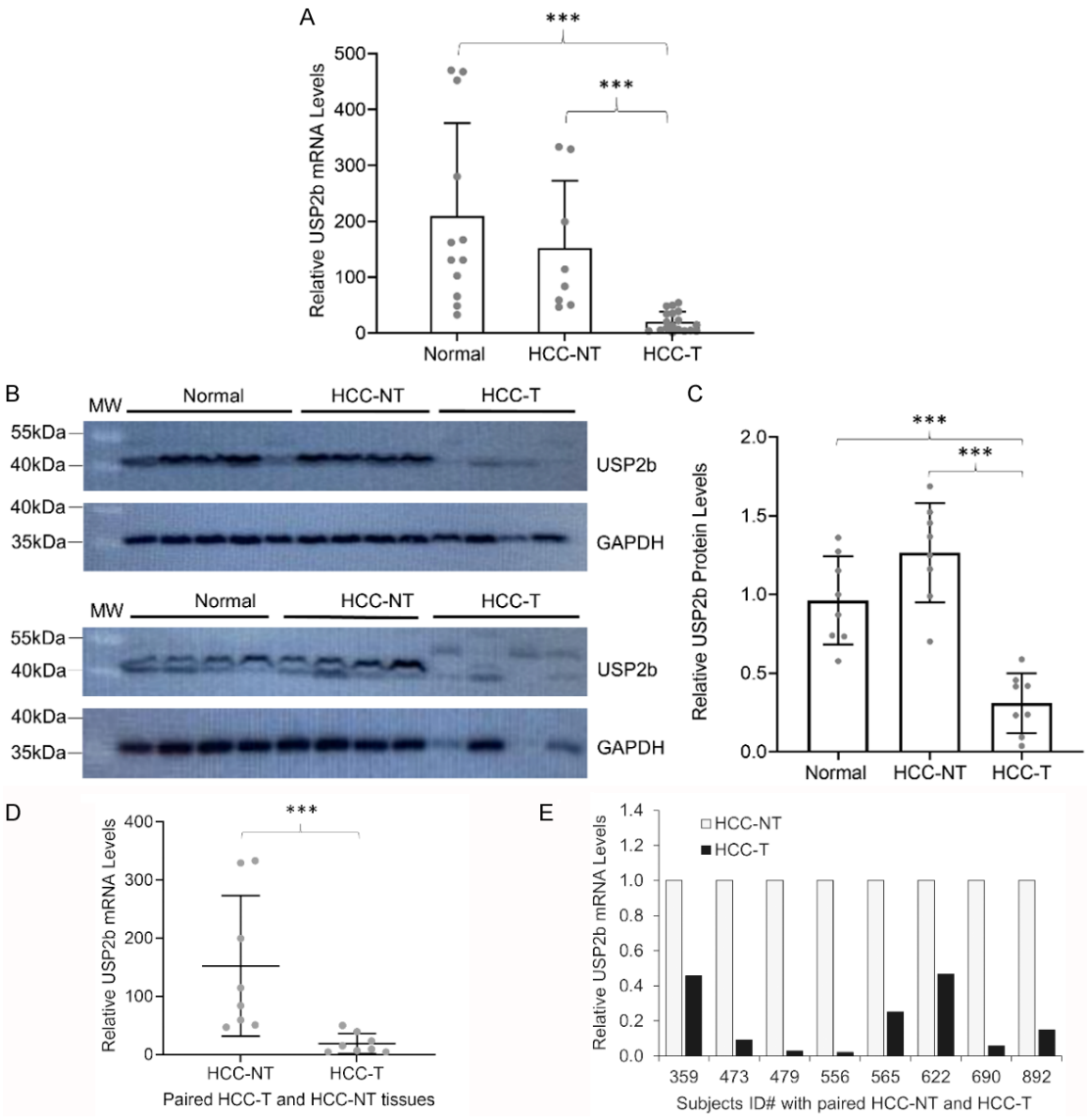


Figure 5. USP2b mRNA expression levels were markedly reduced in HCC-T tissues from HCC patients. (A) The expression levels of USP2 mRNA in human normal liver (n=12), HCC-NT (n=8) and HCC-T (n=21) detected by real-time PCR. (B) USP2b protein detected by Western blot in normal liver (n=8), HCC-NT (n=8) and HCC-T tissues (n=8). (C) Quantification of the USP2b protein levels in (B) by ImageJ. (D) The expression levels of USP2 mRNA in paired HCC-NT and HCC-T tissues as groups as well as (E) individual pairs (n=8). ***P<0.001 in students' t-test for pair-wise comparison or one-way ANOVA, followed by Tukey post-hoc test for multiple comparisons.

was markedly decreased in HCC-T tissues (n=26) when compared to normal (n=16) and HCC-NT tissues (n=26) (**Figure 6A**). In addition, the expression levels of Usp2b in HCC-NT tissues were significantly reduced when compared to that in the normal liver tissues. Of the 26 pairs of HCC-T and HCC-NT samples, 22 samples had decreased Usp2b expression within the tumor section, with 19 samples showing a greater than 20% reduction and 13 samples having greater than a 50% reduction (**Figure 6B**). Consistent with the results of Usp2b mRNA expression, Usp2b protein levels were significantly decreased in HCC-T and HCC-NT when compared to the normal tissues (**Figure 6C** and **6D**). The protein levels were

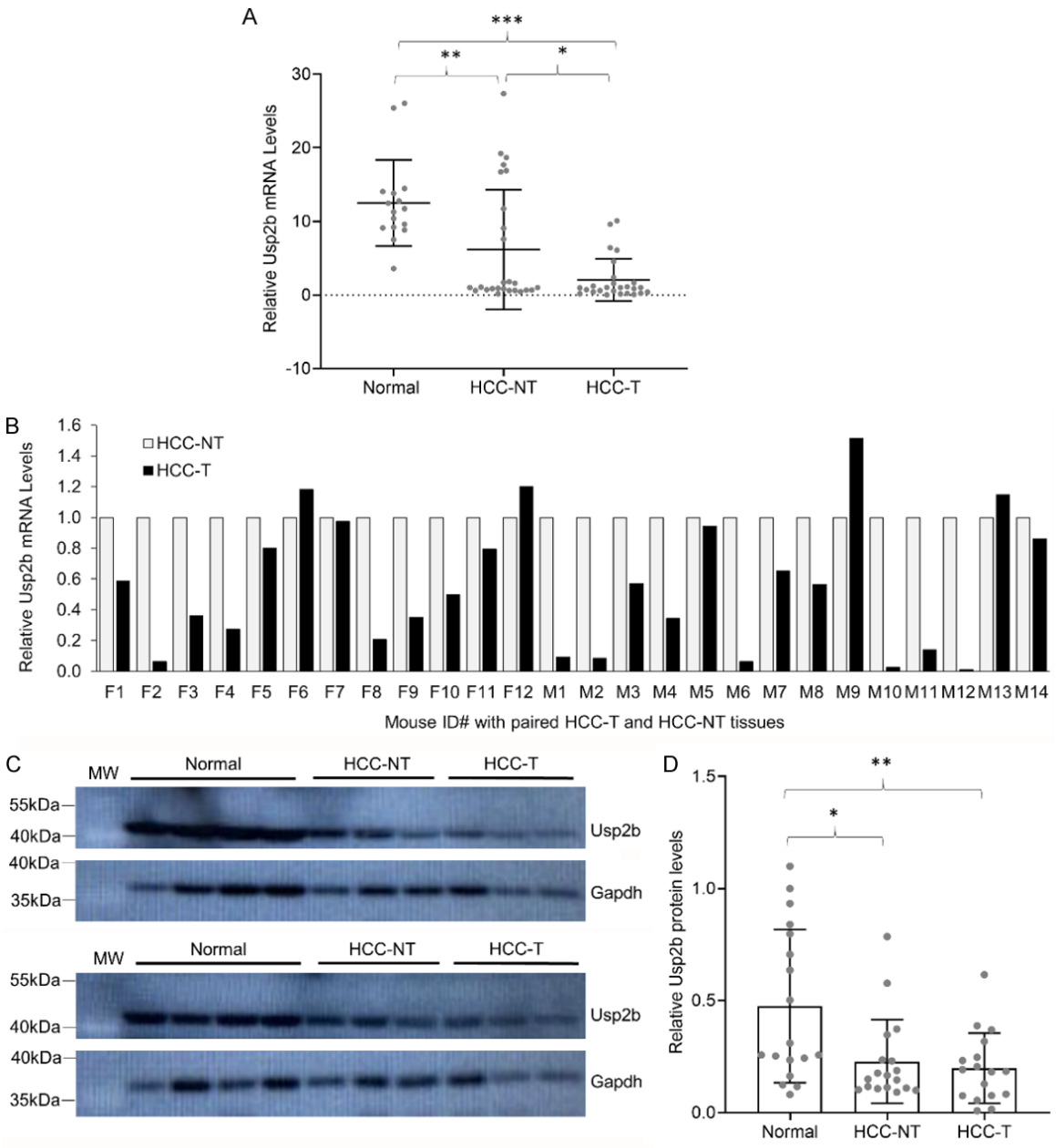


Figure 6. Usp2b mRNA expression levels were significantly deceased in HCC-T tissues from HCC mice. (A) The expression levels of Usp2b mRNA in mouse normal liver (n=16), HCC-NT (n=26) and HCC-T (n=26) samples. (B) The expression levels of Usp2b mRNA in paired HCC-T and HCC-NT tissues (n=26). (C) Representative Western blots of Usp2b protein in normal liver (n=18), HCC-NT (n=18) and HCC-T tissues (n=17). (D) Quantification of Usp2b protein in (C) by ImageJ. *P<0.05, **P<0.01 and ***P<0.001 in one-way ANOVA, followed by Tukey post-hoc test for multiple comparisons.

slightly reduced in HCC-T when compared to HCC-NT tissues without reaching a statistical significance.

USP2b promoted cell proliferation

To determine if USP2b is involved in regulating cell proliferation, a series of cell proliferation assays were carried out. Over-expression of USP2b in HepG2 and Huh 7 cells significantly increased cell growth 24 and 48 h post-transfection (**Figure 7A** and **7B**) (P<0.05, P<0.01 or P<0.001). Consistently, inhibition of USP2 with a selective USP2 inhibitor ML364 dose-dependently reduced HepG2 (**Figure 7C**) and Huh 7 (**Figure 7D**) cell proliferation. At a dose

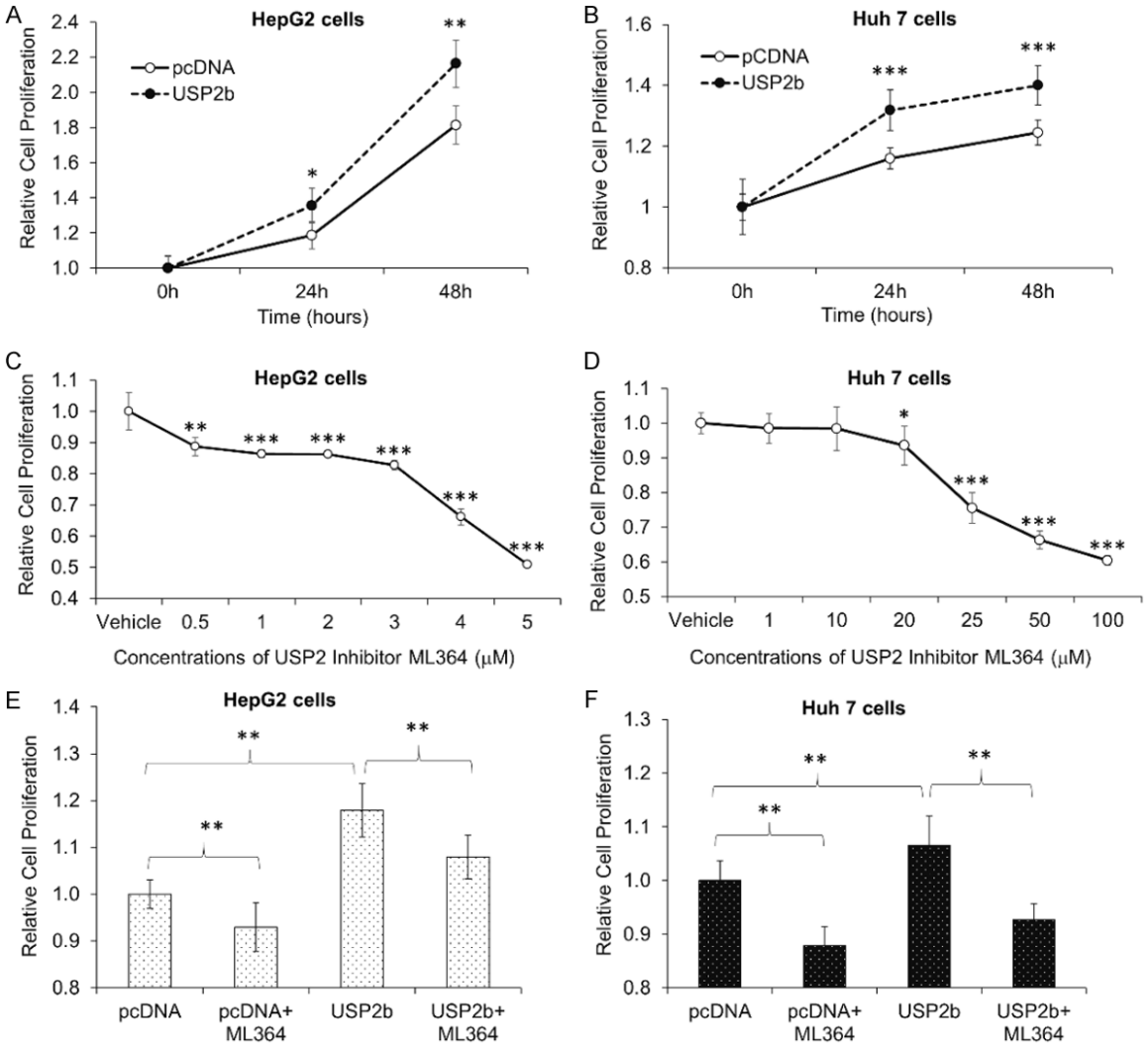


Figure 7. USP2b promoted cell proliferation. (A) HepG2 and (B) Huh 7 cells were transfected with USP2b or pcDNA vector, followed by monitoring cell growth for a period of 48 h by the MTS assays. (C) HepG2 and (D) Huh 7 cells were treated with increasing concentrations of USP2-specific inhibitor ML364 for 48 h, followed by monitoring cell proliferation by MTS assays. (E) HepG2 and (F) Huh 7 cells were transfected with USP2b or pcDNA vector in the presence or absence of USP2 inhibitor ML364 for 48 h, followed by monitoring cell proliferation by MTS assays. *P<0.05, **P<0.01, ***P<0.001 in students' t-test for pair-wise comparison or one-way ANOVA, followed by Tukey post-hoc test for multiple comparisons.

as low as 0.5 μM, the growth of HepG2 cells was significantly inhibited by ML364 (P<0.01). On the other hand, 20 μM ML364 was required for significantly inhibiting Huh 7 cell growth. The inhibitory effects of HepG2 and Huh 7 cell growth by ML364 were further confirmed in the cells transfected with USP2b or pcDNA vector. In the presence of ML364, USP2b-promoted cell proliferation was largely or totally diminished in HepG2 (**Figure 7E**) and Huh 7 cells (**Figure 7F**).

USP2b increased colony formation and promoted wound healing

Consistent with USP2b's ability to promote cell proliferation, over-expression of USP2b in HepG2 and Huh 7 cells significantly increased colony formation (P<0.01) (**Figure 8A** and **8B**). In HepG2 cells, colony formation increased from 28.7 in cells transfected with pcDNA to 77.8 in cells transfected with USP2b (**Figure 8A**). In Huh 7 cells, colony formation increased

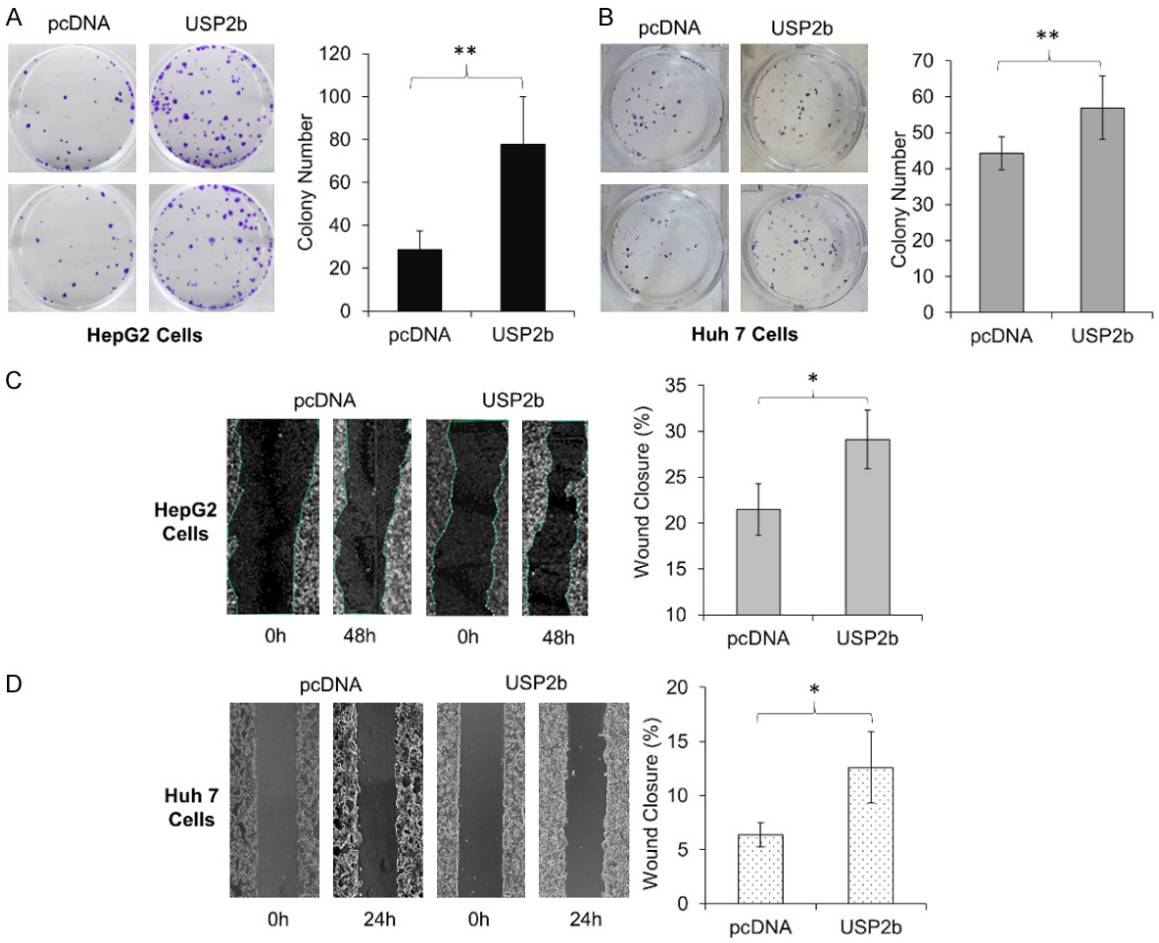


Figure 8. USP2b promoted colony formation and wound-healing. (A) HepG2 and (B) Huh 7 cells were transfected with USP2b or pcDNA vector and then were seeded at a low density in 6-well plates and allowed to grow for 2 weeks for colony formation. The colonies were stained and counted. (C) HepG2 and (D) Huh 7 cells were transfected with USP2b or pcDNA vector. After the cells grew monolayers, a wound gap between the cells was generated by either scratching the cell monolayers or removing the insert in the wells. Images were taken at time of initial wound and 24 or 48 hours post wound formation. The average wound closure percentages were calculated based on the measurements from 3 random fields. *P<0.05 and **P<0.01 in students' t-test for pair-wise comparison.

from 44.3 in pcDNA-transfected cells to 56.9 in USP2b-transfected cells (**Figure 8B**).

Over-expression of USP2b also enhanced wound healing in both HepG2 and Huh 7 cells (**Figure 8C** and **8D**). In HepG2 cells, a 21.3% and 29.3% wound closure were measured in pcDNA and USP2b transfected cells between initial wound infliction and 48 h post (P<0.05) (**Figure 8C**). In Huh 7 cells, a 6.4% and 12.6% wound closure were detected in pcDNA and USP2b-transfected cells 24 h post-wound formation (P<0.05) (**Figure 8D**).

USP2b promoted bile acid-induced apoptosis and necrosis

HCC development is often preceded by various liver injuries with concurrent elevation of hepatic bile acids. Elevated bile acids induce cell death and are directly linked to the development and progression of HCC [26-29]. Bile acids-induced cell death can be categorized into two types, apoptosis [30, 31] and necrosis [32, 33]. Indeed, treatment of HepG2 and Huh 7 cells with 400 μM CDCA resulted in apoptotic and necrotic cell death (**Figure 9**). The peak of apoptotic activities was detected 2 to 4 hours post treatment in HepG2 cells and 4 to 8 hours post-treatment in Huh 7 cells (**Figure 9A** and **9B**). On the other hand, the peak of necrotic activities was detected 12 to 48 hours post-treatment in both HepG2 and Huh 7 cells (**Figure 9C** and **9D**).

To evaluate the effects of USP2b on bile acid-induced apoptosis and necrosis, USP2b was

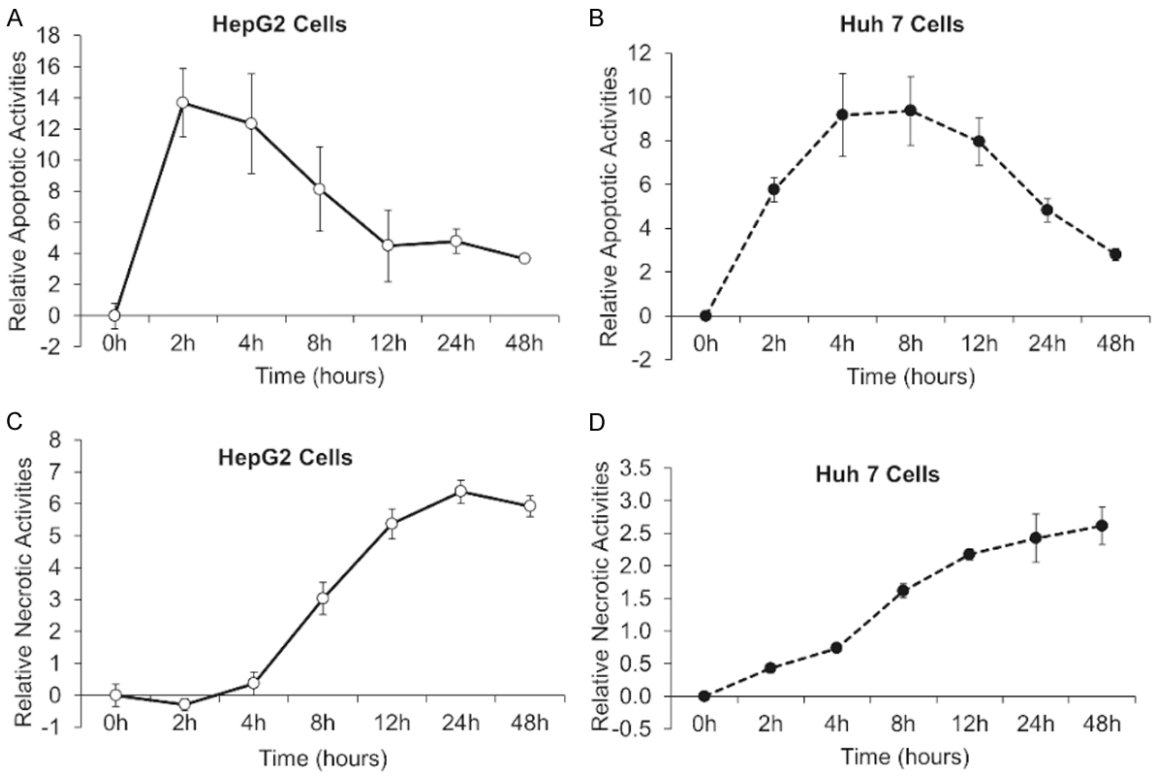


Figure 9. Bile acid CDCA induced apoptosis and necrosis in HepG2 and Huh 7 cells. (A) HepG2 and (B) Huh 7 cells were treated with 400 μM CDCA, followed by detection of apoptotic activities at varying time points up to 48 h. (C) HepG2 and (D) Huh 7 cells were treated with 400 μM CDCA, followed by detection of necrotic activities at various time points up to 48 h.

transfected into HepG2 and Huh 7 cells, followed by treatment of the transfected cells with increasing concentrations of CDCA. Apoptotic activities were quantified 4 hours post-treatment while necrotic activities were measured 24 hours post-treatment. As shown in **Figure 10A** and **10B**, over-expression of USP2b in HepG2 and Huh 7 cells markedly promoted apoptosis when compared with cells transfected with pcDNA vector ($P<0.01$ or $P<0.001$). On the other hand, over-expression of USP2b significantly promoted necrosis in HepG2 cells while had slightly reduced necrotic activities in Huh 7 cells without reaching a statistical significance (**Figure 10C** and **10D**).

Identification of potential USP2b regulated target proteins

As a deubiquitinating enzyme, USP2 regulates the target proteins through modulating protein stability [9-11, 13]. To identify the potential downstream target proteins of USP2 that mediate the effects on cell proliferation and cell death, we generated USP2 knockout (USP2-KO) cells from parental HepG2 cells, followed by proteomic profiling of USP2-KO and parental HepG2 cells. USP2-KO cells were generated using CRISPR and two independent cell clones USP2-KO (3A) and USP2-KO (3E) were characterized. Consistent with the findings that USP2b promotes cell proliferation (**Figure 7A**), the cell proliferation rates of the two independent USP2-KO cell lines, USP2-KO (3A) and USP2-KO (3E), were significantly reduced compared to the parental HepG2 cells (**Figure 11A** and **11B**). The two USP2-KO cell clones exhibited almost identical cell growth characteristics over the period of 96 hours. As expected, over-expression of USP2b in both USP2-KO cells increased cell growth (**Figure 11C** and **11D**).

Proteomic profiling was carried out using the parental HepG2, USP2-KO (3A) and USP2-KO (3E) cells. As shown in **Figure 12A**, among the 4861 proteins subjected for quantitative analysis, a total of 210 and 149 proteins were significantly increased and decreased in abundance

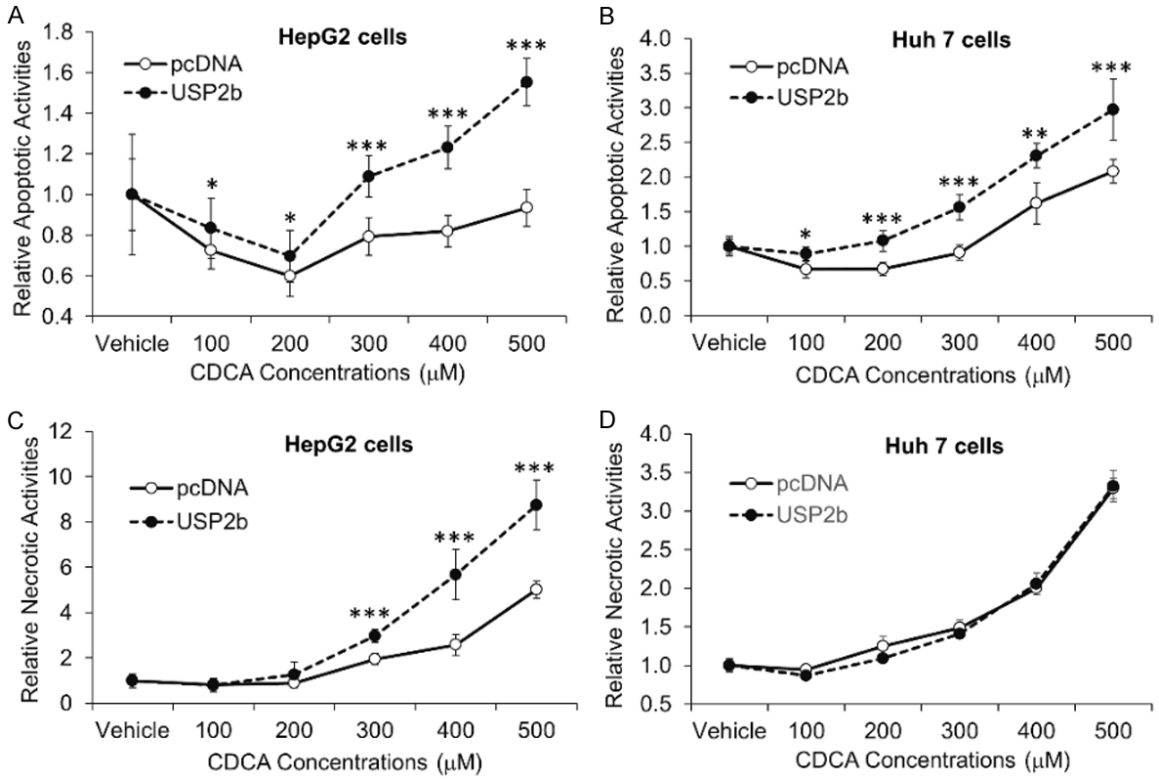


Figure 10. USP2b promoted bile acid-induced apoptosis and necrosis. (A) HepG2 and (B) Huh 7 cells were transfected with USP2b or pcDNA vector and the transfected cells were treated with increasing concentrations of CDCA or vehicle for 2-4 h, followed by detection of the apoptotic activities. (C) HepG2 and (D) Huh 7 cells were transfected with USP2b or pcDNA vector and the transfected cells were treated with increasing concentrations of CDCA or vehicle for 12-24 h, followed by detection of the necrotic activities. *P<0.05, **P<0.01 and ***P<0.001 in students' t-test for pair-wise comparison.

in USP2-KO (3A) cells compared to the parental HepG2 cells (1.5 fold up or down at q<0.05), respectively. Comparably, a total of 178 and 146 proteins were significantly increased and decreased in abundance in USP2-KO (3E) cells when compared to the parental HepG2 cells (1.5 fold up or down at q<0.05), respectively (**Figure 12B**). Among the altered proteins, 190 proteins (more than 50%) overlapped between the two cell clones (**Figure 12C**). Gene enrichment and pathway analysis of these 190 proteins revealed many cellular functional pathways, including lipid metabolism, protein modifications, cell metabolism and regulation, programmed cell death/apoptosis and regulation, cell activation, proliferation and differentiation (**Figure 12D**).

Analysis of the up-regulated proteins revealed that 126 proteins overlapped between the two cell clones (**Figure 12E**). The signaling pathways or cellular processes enriched included metabolism and regulation, protein modifications, immune and inflammation responses, cell activation and proliferation (**Figure 12F**). Analysis of down-regulated proteins showed that 64 proteins were overlapped between the two cell clones (**Figure 12G**). The shared proteins were enriched in programmed cell death/apoptosis and regulation, responses to wound healing and regulation, liver development and regeneration, cell growth and lipid metabolism (**Figure 12H**).

Among the up-regulated proteins, we identified eight proteins involved in cell proliferation/growth, cell death/apoptosis and the pathogenesis of cancer (**Figure 12I** and **Table 1**), including rho-associated coiled-coil-containing protein kinase 1 (ROCK1), protein kinase N1 (PKN1), transcription factor 3 (TCF3), integrin subunit beta 3 (ITGB3), AlkB homolog 5 RNA demethylase (ALKBH5), pelota mRNA surveillance and ribosome rescue factor (PELO), ste-

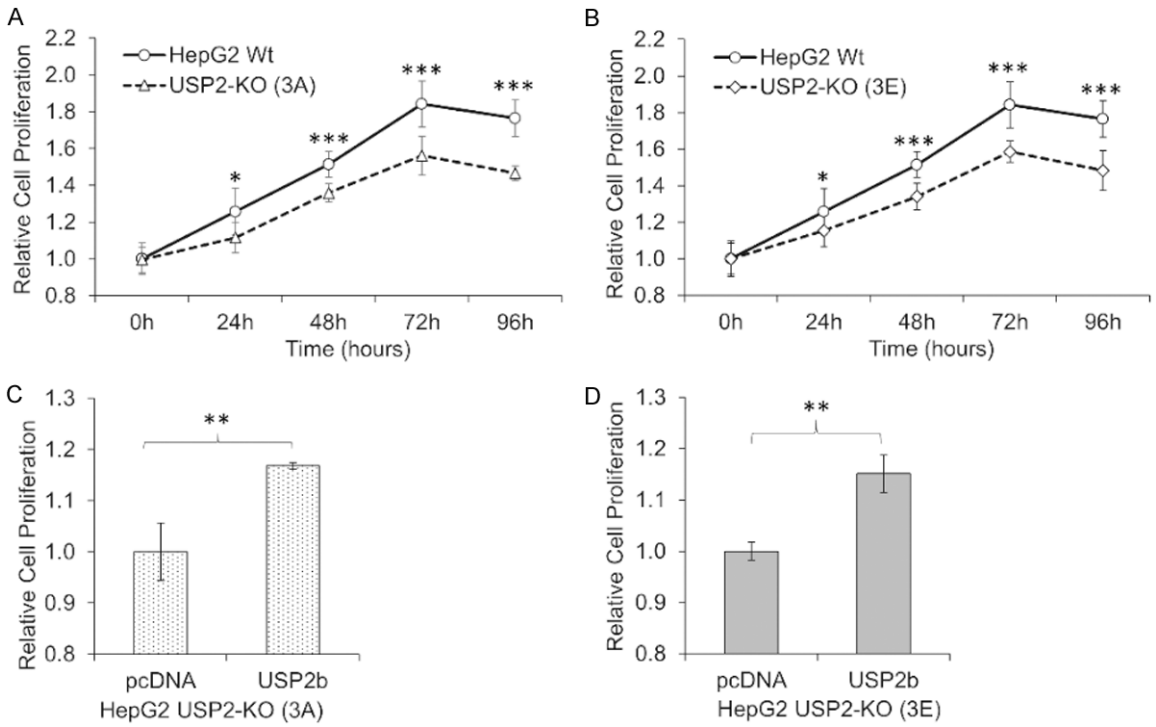


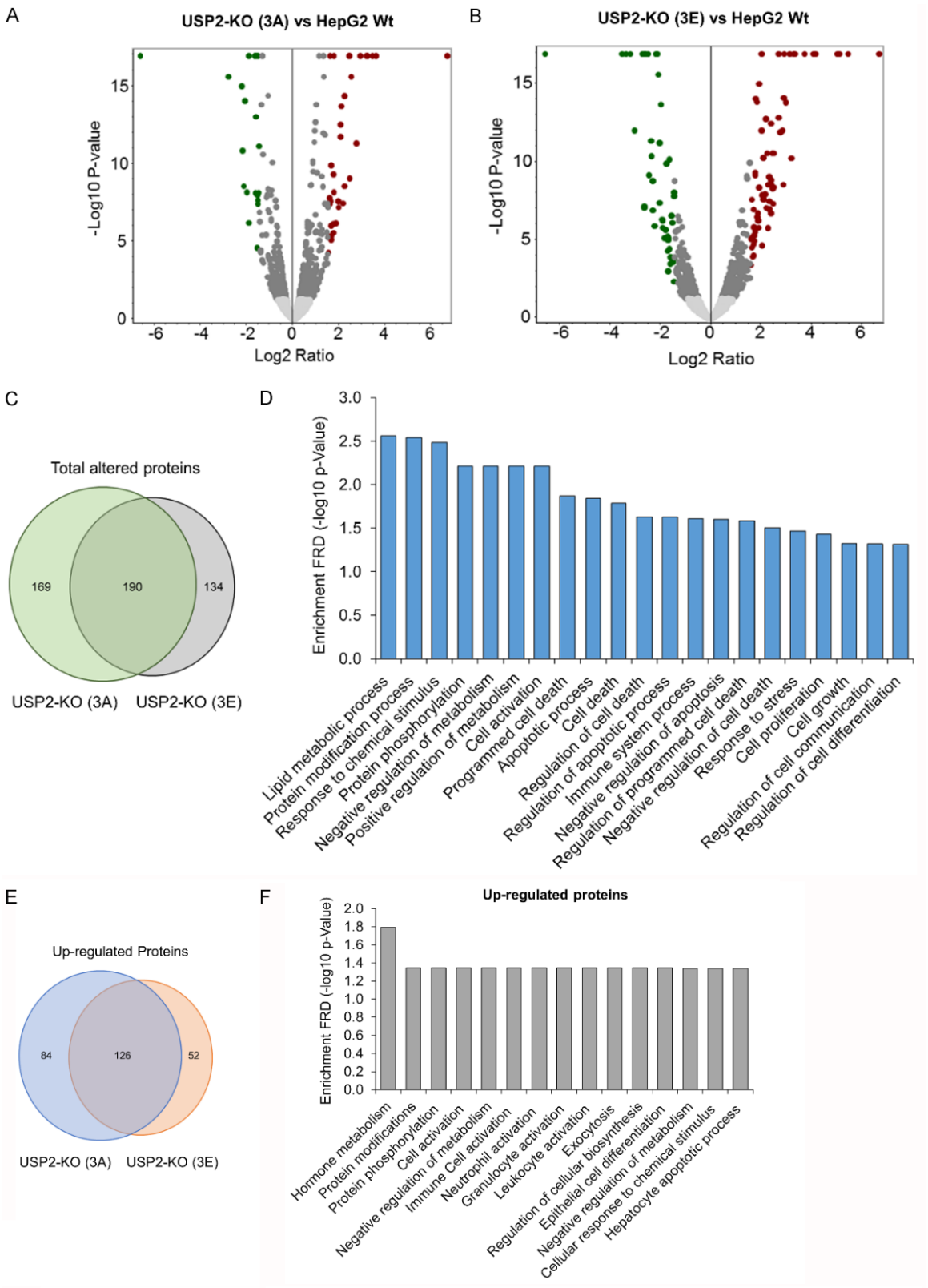
Figure 11. Knockout of USP2 significantly reduced cell proliferation. (A) Relative proliferation of parental HepG2 and USP2-KO (3A) and (B) USP2-KO (3E) cells over a period of 96 h. (C) USP2-KO (3A) and (D) USP2-KO (3E) cells were transfected with USP2b or pcDNA vector, followed by monitoring the cell proliferation over a period of 48 h. *P<0.05, **P<0.01 and ***P<0.001 in students' t-test for pair-wise comparison.

roid receptor RNA activator 1 (SRA1) and serine/threonine kinase 4 (STK4). Among the down-regulated proteins, six proteins involved in cell proliferation, apoptosis and tumorigenesis were identified including glycogen synthase kinase 3 beta (GSK3B), autophagy related 3 (ATG3), fatty acid binding protein 1 (FABP1), dipeptidyl peptidase 4 (DPP4), epidermal growth factor receptor (EGFR) and heme oxygenase 1 (HMOX1) (**Figure 12I** and **Table 1**).

The proteomic data was further validated by immunoblot analysis of the four proteins of interest including EGFR, STK4, DPP4 and FABP1. As shown in **Figure 13A**, consistent with the proteomic results, STK4 protein levels were significantly up-regulated in both USP2-KO (3A) and USP2-KO (3E) cells when compared to HepG2 wt cells. On the other hand, the expression levels of FABP1, DPP4 and EGFR proteins were significantly down-regulated in both USP2-KO (3A) and USP2-KO (3E) cells when compared with HepG2 wt cells (**Figure 13B-D**), confirming the data from the proteomic profiling (**Figure 12E** and **Table 1**).

Discussion

USP2 has been implicated in various diseases, particularly multiple types of cancers, with majority of the studies being focused on USP2a. In prostate cancer and glioma, USP2a expression was upregulated [8, 34, 35]. On the other hand, in bladder cancer and renal cell carcinoma, USP2a was markedly downregulated [36, 37]. In breast cancer, both up- and down-regulation of USP2 were reported [19, 38]. Limited studies have been done to determine whether USP2 expression is dysregulated in HCC or its roles in HCC pathogenesis. USP2a expression was reported to be upregulated in a large portion of the HCC tumor tissues while USP2b was not investigated [18, 39]. In this study, we demonstrated that USP2 expression was dysregulated both at mRNA and protein levels in patients with HCC. Compared with normal liver tissues, USP2 expression in HCC-T was significantly reduced at both protein and mRNA levels (**Figures 1** and **2**). Consistent with the findings from human, USP2 expression was also significantly down-regulated in HCC-T in the FXR-KO


A
USP2-KO (3A) vs HepG2 Wt
-Log10 P-value
Log2 Ratio
B
USP2-KO (3E) vs HepG2 Wt
-Log10 P-value
Log2 Ratio
C
Total altered proteins
169
190
134
USP2-KO (3A)
USP2-KO (3E)
D
Enrichment FRD (-log10 p-Value)
Lipid metabolic process
Protein modification process
Response to chemical stimulus
Protein phosphorylation
Negative regulation of metabolism
Positive regulation of metabolism
Cell activation
Programmed cell death
Apoptotic process
Cell death
Regulation of cell death
Regulation of apoptotic process
Immune system process
Negative regulation of apoptosis
Regulation of programmed cell death
Negative regulation of cell death
Response to stress
Cell proliferation
Cell growth
Regulation of cell communication
Regulation of cell differentiation
E
Up-regulated Proteins
84
126
52
USP2-KO (3A)
USP2-KO (3E)
F
Up-regulated proteins
Enrichment FRD (-log10 p-Value)
Hormone metabolism
Protein modifications
Protein phosphorylation
Cell activation
Negative regulation of metabolism
Immune Cell activation
Neutrophil activation
Granulocyte activation
Leukocyte activation
Exocytosis
Regulation of cellular biosynthesis
Epithelial cell differentiation
Negative regulation of metabolism
Cellular response to chemical stimulus
Hepatocyte apoptotic process

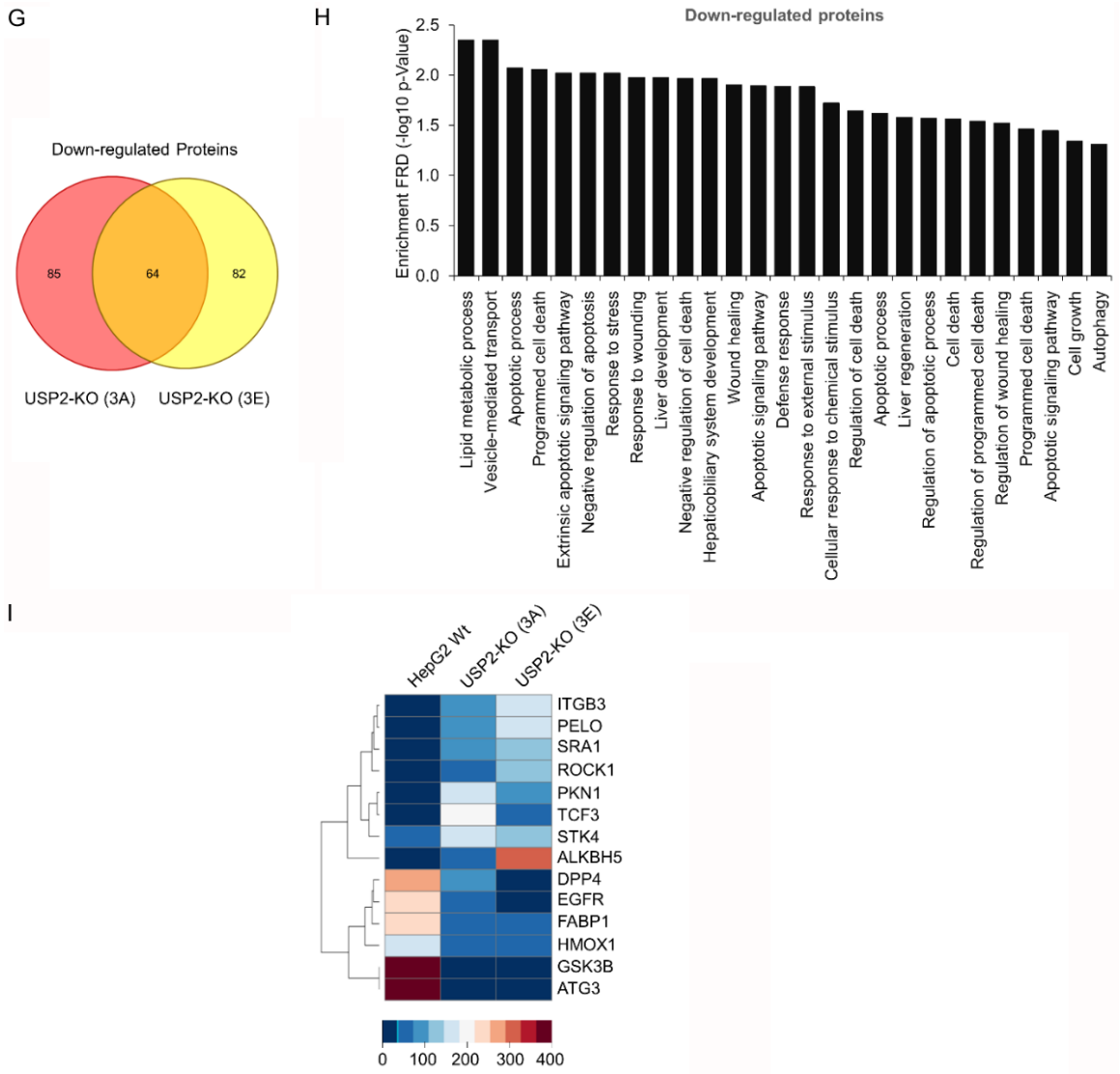


Figure 12. Identification of USP2-regulated target proteins with proteomic profiling. (A) Quantitative analysis of the proteomics dataset obtained from parental HepG2 (n=3 duplicates) and USP2-KO (3A) cells (n=3 duplicates) and (B) from HepG2 wt and USP2-KO (3E) cells (n=3 duplicates). Volcano plot shows the fold-change (1.5 fold up or down) versus *p*-value of protein abundance for the total of 4861 proteins identified. Red and green circles represent the significant (q<0.05) up- and down-regulated proteins in USP2-KO (3A) or USP2-KO (3E) compared with the parental HepG2 cells. (C) Overlap of the proteins differentially expressed in USP2-KO (3A) and USP2-KO (3E) cells when compared with the parental HepG2 cells. (D) The protein enrichment analysis of the overlapped altered proteins by KEGG and ShinyGo. (E) Overlap of upregulated proteins differentially expressed in USP2-KO (3A) and USP2-KO (3E) cells when compared with HepG2 cells. (F) The protein enrichment analysis of the overlapped upregulated proteins by KEGG and ShinyGo. (G) Overlap of downregulated proteins differentially expressed in USP2-KO (3A) and USP2-KO (3E) cells when compared with parental HepG2 cells. (H) The protein enrichment analysis of the overlapped downregulated proteins by KEGG and ShinyGo. (I) The heat map analysis of the USP2 regulated target proteins associated with cell proliferation, apoptosis, and tumorigenesis in the parental HepG2, USP2-KO (3A) and USP2-KO (3E) cells.

mouse HCC model (**Figure 3**). Most previous studies with various cancers used the adjacent tissues as normal controls for gene expression comparison with tumor tissues. The advantage of such comparison is to minimize the individual variations. However, the adjacent tissues may not represent the actual normal status of the tissues. Indeed, our results revealed that USP2 expression levels exhibited a reduced trend from normal to HCC-NT to HCC-T in both

Table 1. Up- and down-regulated proteins in USP2-KO (3A) and USP2-KO (3E) cells when compared to parental HepG2 cells

Up-regulated proteins				
Proteins	USP2-KO (3A) vs HepG2 cells (Fold increase)	*p* value	USP2-KO (3E) vs HepG2 cells (Fold increase)	*p* value
ROCK1	100	3.10×10^{-16}	100	2.67×10^{-16}
PKN1	100	3.10×10^{-16}	100	2.67×10^{-16}
TCF3	100	3.10×10^{-16}	100	2.67×10^{-16}
ITBG3	4.20	5.17×10^{-13}	8.58	2.67×10^{-16}
ALKBH5	6.55	1.27×10^{-10}	33.92	2.67×10^{-16}
PELO	3.77	5.56×10^{-7}	7.24	1.74×10^{-13}
SRA1	3.57	1.28×10^{-5}	4.87	1.81×10^{-6}
STK4	3.28	1.36×10^{-5}	2.80	4.44×10^{-3}
Down-regulated proteins				
Proteins	USP2-KO (3A) vs HepG2 cells (% reduction)	*p* value	USP2-KO (3E) vs HepG2 cells (% reduction)	*p* value
GSK3B	99.0	3.10×10^{-16}	99.0	2.67×10^{-16}
ATG3	99.0	3.10×10^{-16}	99.0	2.67×10^{-16}
FABP1	68.8	3.10×10^{-16}	78.8	2.67×10^{-16}
DPP4	66.1	3.10×10^{-16}	76.6	1.30×10^{-10}
EGFR	77.7	6.14×10^{-8}	99.0	2.67×10^{-16}
HMOX1	43.7	3.59×10^{-4}	50.1	0.022

human (**Figure 2A**) and mice (**Figure 3A**). The expression levels of USP2 in HCC-NT were significantly reduced when compared to normal liver tissue in mice (**Figure 3A**). Our data support the notion that HCC-NT tissues are different from normal and HCC-T tissues and represents the intermediate status from normal to tumor tissues [40, 41].

Three USP2 isoforms including USP2a, USP2b and USP2c have been identified from various organs [13-15] with USP2a being most studied. Currently, the study on USP2 isoforms in the liver is limited and controversial [14, 16, 17]. In one study, it was reported that USP2c (USP-41KD) was the predominant USP2 isoform (89% of total USP2) while no USP2b proteins were detected [16]. In contrast, other studies reported that USP2b was the predominant isoform while USP2c was not detected [14, 17]. In this study, we found that USP2a and USP2b were detected in both human and mouse liver while USP2c was under detection by real-time PCR (**Figure 4A** and **4B**). Compared with USP2a, USP2b is much more abundantly expressed in both human (**Figure 4A**) and mouse liver (**Figure 4B**). Consistent with USP2b being the predominant isoform of USP2, the expression profiles of USP2b resembled that of total USP2 with a similar downtrend from normal to HCC-NT to HCC-T tissues in both human (**Figures 2A** and **5**) and mice (**Figures 3A** and **6**). Taken together, these data demonstrated that USP2b was the predominant USP2 isoform in the liver and exhibited a consistent downtrend from normal to HCC-NT to HCC-T tissues in both human and mice.

Previous studies on USP2's involvement in regulating cell proliferation, apoptosis and invasion have been limited to USP2a or USP2c [8-11]. In this study, we found that USP2b promoted cell proliferation in HepG2 (**Figure 7A** and **7E**), Huh 7 (**Figure 7B** and **7F**) and USP2-KO cells (**Figure 11C** and **11D**). Consistently, overexpression of USP2b promoted colony formation and wound healing in both HepG2 (**Figure 8A** and **8C**) and Huh 7 cells (**Figure 8B** and **8D**). On the other hand, inhibition of USP2 activity by the chemical inhibitor ML364 or knockout of USP2 in USP2-KO cells significantly reduced cell proliferation (**Figures 7E**, **7F**, **11A**, **11B**). The results thus firmly established that USP2b promoted cell proliferation, thus contributing to the pathogenesis of HCC.

HCC is often developed from chronic liver disorders such as cholestasis, hepatitis, and cirrho-

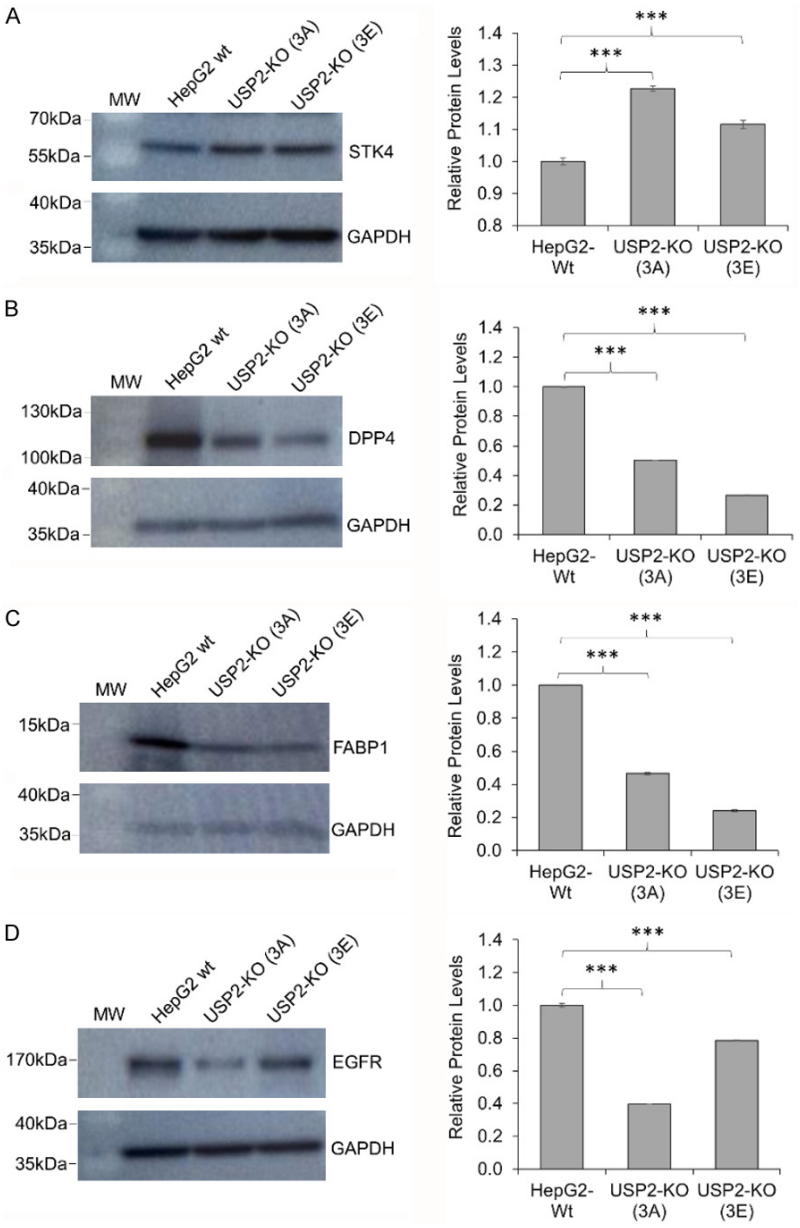


Figure 13. Confirmation of USP2-regulated target proteins by Western blotting. (A) Detection and quantification of USP2-regulated target protein STK4, (B) DPP4, (C) FABP1 and (D) EGFR in parental HepG2, USP2-KO (3A) and USP2-KO (3E) cells. ***P<0.001 with one-way ANOVA, followed by Tukey post-hoc test for multiple comparisons.

sis. Increased intrahepatic bile acid levels as a result of liver disorders are linked to the development and progression of HCC [26-29]. Young children carrying genetic defects on bile salt export pump (BSEP) have elevated bile acids and develop HCC at early ages [27]. Consistently, elevated bile acid concentrations play a role in promotion and progression of HCC in rodents [28, 42]. It is well established that high levels of bile acids are toxic to hepatocytes, causing cell death through either apoptosis [30, 31] or necrosis [32, 33]. In this study, we investigated the effects of USP2b on bile acid-induced apoptosis and necrosis. We found that USP2b significantly promoted bile acid-induced apoptosis (**Figure 10A** and **10B**) and necrosis (**Figure 10C**), thus playing a role in the pathogenesis of HCC. Our results are consistent with the proapoptotic activity of USP2b detected in HEK293 cells [43].

Taken together, our data demonstrated that USP2b exhibited both tumor-suppressing and promoting activities. On the one hand, USP2b expression was significantly down-regulated in HCC tumors from both human and mouse, and overexpression of USP2b enhanced bile acids-induced apoptosis and necrosis, showing the characteristics of a tumor suppressor. On the other hand, USP2b promoted cell proliferation, colony formation and wound healing, displaying the features of an oncogene. Such phenomenon is not uncommon. A number of other proteins have been reported to have both oncogenic and tumor-suppressing activities, including transforming growth factor-beta (TGF-β) [44, 45], Sirtuin 1 (SIRT1) [46], nuclear receptor 4A (NR4A) [47], high mobility group box protein 1 (HMGB1) [48], yin yang 1 (YY1) [49] and many others [50-53]. Most of those proteins are either transcriptional factors or epigenetic modifying enzymes which regulate many downstream targets and signaling circuits. Those proteins can function as tumor suppressor or

tumor promoter depending on the tumor stages, specific organs, cell types, and specific cellular conditions. Similarly, as a deubiquitinating enzyme, USP2 regulates a large number of downstream targets involved in cell proliferation, apoptosis or necrosis (**Figure 12**). Therefore, USP2b's function as a tumor suppressor or promoter may depend on the cell status or context. In the presence of elevated hepatic bile acids *in vivo* in HCC subjects and high levels of bile acids *in vitro* during apoptosis and necrosis assays, USP2b may predominantly act as a tumor suppressor. However, in the absence of bile acids *in vitro* during cell proliferation, colony formation and wound healing assays, USP2b may predominately function as a cell proliferation promoter. Consistent with its function as a tumor suppressor, USP2b expression was significantly reduced in the HCC tumor. Reduced expression of USP2b made the tumor cells more resistant to the apoptotic and necrotic cell death induced by elevated bile acids in the HCC subjects, thus favoring tumor cell survival. However, it remains to be determined whether reduced USP2b expression results in slower growth of the HCC tumor *in vivo* in the presence of elevated hepatic bile acids.

USP2b's ability to promote cell proliferation appears to contradict with its pro-apoptotic and pro-necrotic activities. One possible explanation is that USP2b promotes cell proliferation or cell-death dependent on the specific cellular conditions as stated above. The second possible explanation for such phenomenon is that USP2b promotes apoptotic cell death and cellular factors released from the dying cells subsequently promote surrounding cell proliferation. Indeed, cell death through apoptosis has been proposed as the driving force for increased cell proliferation [54-56]. Such apoptosis-induced proliferation is considered beneficial for the organism since it allows damaged tissues to be repaired or regenerated. Therefore, in addition to its roles in the HCC pathogenesis, USP2b may also play an important role in liver damage repairing and regeneration.

In this study, through an unbiased proteomic approach, we identified USP2-regulated target proteins involved in cell proliferation/growth, apoptosis and necrosis. Among those targets, four proteins including STK4, EGFR, DPP4 and FABP1 have been notably connected to the pathogenesis of HCC and other cancers. EGFR, DPP4 and FABP1 protein levels were significantly reduced in USP2-KO compared to HepG2 wt cells (**Figures 12I**, **13B-D** and **Table 1**), which is in line with the notion that USP2 stabilizes its target proteins through deubiquitination preventing ubiquitin-mediated protein degradation. Serving as the receptor for epidermal and other growth factors, EGFR has been long recognized to exert a variety of cellular activities in cell proliferation, differentiation, survival and tumorigenesis [57-59]. Dysregulation of EGFR signaling pathways leads to increased cell proliferation, differentiation and eventually HCC development. Consistent with our findings is a report that USP2a regulated EGFR degradation through deubiquitination in lung cancer [60]. DPP4 exerts a myriad of functional activities through processing various target peptides. Several lines of evidence firmly connect DPP4 to HCC development [61-64]. However, it is still not clear whether DPP4 functions as a tumor suppressor or promoter. Earlier studies showed that DPP4 expression was upregulated in HCC patients and inhibition of DPP4 reduced or prevent HCC development in mice [62-64], indicating that DPP4 acts as an oncogene. However, a most recent study with a larger sample size reported that DPP4 protein expression was significantly downregulated in HCC patients and reduced DPP4 protein expression directly correlated with poor prognosis [61], displaying the features of a tumor suppressor. However, it should be emphasized that in contrast to protein levels, DPP4 mRNA levels did not show any correlations with HCC prognosis [61], indicating the importance of post-transcriptional regulation of DPP4, which is consistent with regulatory effects of USP2 on DPP4 protein stability. FABP1 plays important roles in regulating lipid metabolism, energy homeostasis, and antioxidant responses [65]. Dysregulation of liver FABP1 is implicated in various liver disorders including cholestasis, steatosis, non-alcoholic fatty liver disease and HCC [65]. Dysregulated expression of FABP1 correlated with tumorigenesis or prognosis in HCC subjects [65-67]. Taken together, EGFR, DPP4 and FABP1 all exhibit activities associated with HCC development and progression. It is reasonable to speculate that USP2 mediated its activities through upregulating EGFR, DPP4 and FABP1 protein expression. As one of the USP2 target proteins,

STK4 acts as a tumor suppressor, exerting its activities mainly through the Hippo/Yap1 and Toll-like receptor (TLR) signaling pathway [68, 69]. Deficiency of STK4 in the liver results in massive liver overgrowth and HCC development in mice [70]. In contrast to EGFR, DPP4 and FABP1, STK4 protein expression was significantly elevated in USP2-KO cells compared to HepG2 wt cells (**Figures 12I**, **13A** and **Table 1**), indicating that USP2 down-regulates STK4 protein expression. Such down-regulation of STK4 protein by USP2 contrasts with the generally accepted roles of USP2 in stabilizing target proteins. One possible explanation is that USP2 may directly promote STK4 protein degradation. Consistent with this notion, USP2 directly deubiquitinates its target immune deficiency (Imd) protein and promotes its degradation through the proteasomal pathway [71]. The other possible explanation is that USP2 regulates STK4 protein expression indirectly. Taken together, USP2 may exert its activities in cell proliferation and death through regulating STK4, EGFR, DPP4 and FABP1.

In summary, USP2 and its predominant isoform USP2b were dysregulated in HCC tissues from both human and mice. Such dysregulation of USP2b contributes to the pathogenesis of HCC through promoting cell proliferation as well as exerting proapoptotic and pronecrtotic activities. USP2b's ability to act as tumor suppressor or promoter depend on the cell status or context. We are currently investigating the underlying mechanisms by which USP2b promotes cell growth while exerts proapoptotic and pronecrtotic activities. The findings from the current study provide the molecular basis for developing therapies for HCC through modulating USP2b expression or activities.

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Disclosure of conflict of interest

None.

Abbreviations

ALKBH5, AlkB homolog 5 RNA demethylase; ATCC, American Type Culture Collection; ATG3, autophagy related 3; CDCA, chenodeoxycholic acid; Ct, cycle threshold; DPP4, dipeptidyl peptidase 4; DUB, deubiquitinating enzymes; EGFR, epidermal growth factor receptor; FABP1, fatty acid binding protein 1; FDR, false discovery rate; GAPDH, glyceraldehyde 3-phosphate dehydrogenase; GSK3B, glycogen synthase kinase 3 beta; HCC, hepatocellular carcinoma; HCC-NT, HCC adjacent non-tumor tissue; HCC-T, HCC tumor tissue; HMGB1, high mobility group box protein 1; HMOX1, heme oxygenase 1; Imd, immune deficiency; IHC, immunohistochemistry; ITGB3, integrin subunit beta 3; KEGG, Kyoto encyclopedia of genes and genomes; NF-κB, nuclear factor kappa-light-chain-enhancer of activated B cells; NR4A, nuclear receptor 4A; PELO, pelota mRNA surveillance and ribosome rescue factor; PKN1, protein kinase N1; ROCK1, Rho associated coiled-coil-containing protein kinase 1; SIRT1, Sirtuin 1; STK4, serine/threonine kinase 4; SRA1, steroid receptor RNA activator 1; TCF3, transcription factor 3; TGF-β, transforming growth factor-beta; TLR, Toll-like receptor; TNF-α, tumor necrosis factor α; USP2, Ubiquitin specific peptidase 2; USP2a, USP2 isoform a; USP2b, USP2 isoform b; USP2c, USP2 isoform c; USP2-KO, USP2 knockout; YY1, yin yang 1.

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References

[1] Waller LP, Deshpande V and Pyrsopoulos N. Hepatocellular carcinoma: a comprehensive review. World J Hepatol 2015; 7: 2648-2663.

[2] Centers for Disease Control and Prevention, National Center for Health Statistics. Trends in liver cancer mortality among adults aged 25 and over in the United States, 2000-2016. July 2018 (https://www.cdc.gov/nchs/products/databriefs/db314.htm).

[3] Wege H, Li J and Ittrich H. Treatment lines in hepatocellular carcinoma. Visc Med 2019; 35: 266-272.
[4] Komander D and Rape M. The ubiquitin code. Annu Rev Biochem 2012; 81: 203-229.
[5] Jang HH. Regulation of protein degradation by proteasomes in cancer. J Cancer Prev 2018; 23: 153-161.
[6] Mennerich D, Kubaichuk K and Kietzmann T. DUBs, hypoxia, and cancer. Trends Cancer 2019; 5: 632-653.
[7] Hanpude P, Bhattacharya S, Dey AK and Maiti TK. Deubiquitinating enzymes in cellular signaling and disease regulation. IUBMB Life 2015; 67: 544-555.
[8] Tao BB, He H, Shi XH, Wang CL, Li WQ, Li B, Dong Y, Hu GH, Hou LJ, Luo C, Chen JX, Chen HR, Yu YH, Sun QF and Lu YC. Up-regulation of USP2a and FASN in gliomas correlates strongly with glioma grade. J Clin Neurosci 2013; 20: 717-720.
[9] Graner E, Tang D, Rossi S, Baron A, Migita T, Weinstein LJ, Lechpammer M, Huesken D, Zimmermann J, Signoretti S and Loda M. The isopeptidase USP2a regulates the stability of fatty acid synthase in prostate cancer. Cancer Cell 2004; 5: 253-261.
[10] Wang CL, Wang JY, Liu ZY, Ma XM, Wang XW, Jin H, Zhang XP, Fu D, Hou LJ and Lu YC. Ubiquitin-specific protease 2a stabilizes MDM4 and facilitates the p53-mediated intrinsic apoptotic pathway in glioblastoma. Carcinogenesis 2014; 35: 1500-1509.
[11] Mahul-Mellier AL, Datler C, Pazarentzos E, Lin B, Chaisaklert W, Abuali G and Grimm S. Deubiquitinating proteases USP2a and USP2c cause apoptosis by stabilising RIP1. Biochim Biophys Acta 2012; 1823: 1353-1365.
[12] Kitamura H and Hashimoto M. USP2-related cellular signaling and consequent pathophysiological outcomes. Int J Mol Sci 2021; 22: 1209.
[13] Baek SH, Choi KS, Yoo YJ, Cho JM, Baker RT, Tanaka K and Chung CH. Molecular cloning of a novel ubiquitin-specific protease, UBP41, with isopeptidase activity in chick skeletal muscle. J Biol Chem 1997; 272: 25560-25565.
[14] Gousseva N and Baker RT. Gene structure, alternate splicing, tissue distribution, cellular localization, and developmental expression pattern of mouse deubiquitinating enzyme isoforms Usp2-45 and Usp2-69. Gene Expr 2003; 11: 163-179.
[15] Zhu HQ and Gao FH. The molecular mechanisms of regulation on USP2's alternative splicing and the significance of its products. Int J Biol Sci 2017; 13: 1489-1496.
[16] Haimerl F, Erhardt A, Sass G and Tiegs G. Down-regulation of the de-ubiquitinating enzyme ubiquitin-specific protease 2 contributes to tumor necrosis factor-alpha-induced hepatocyte survival. J Biol Chem 2009; 284: 495-504.
[17] Molusky MM, Li S, Ma D, Yu L and Lin JD. Ubiquitin-specific protease 2 regulates hepatic gluconeogenesis and diurnal glucose metabolism through 11β-hydroxysteroid dehydrogenase 1. Diabetes 2012; 61: 1025-1035.
[18] Calvisi DF, Wang C, Ho C, Ladu S, Lee SA, Mattu S, Destefanis G, Delogu S, Zimmermann A, Ericsson J, Brozzetti S, Staniscia T, Chen X, Dombrowski F and Evert M. Increased lipogenesis, induced by AKT-mTORC1-RPS6 signaling, promotes development of human hepatocellular carcinoma. Gastroenterology 2011; 140: 1071-1083.
[19] Metzig M, Nickles D, Falschlehner C, Lehmann-Koch J, Straub BK, Roth W and Boutros M. An RNAi screen identifies USP2 as a factor required for TNF-α-induced NF-κB signaling. Int J Cancer 2011; 129: 607-618.
[20] Yang F, Huang X, Yi T, Yen Y, Moore DD and Huang W. Spontaneous development of liver tumors in the absence of the bile acid receptor farnesoid X receptor. Cancer Res 2007; 67: 863-867.
[21] Kim I, Morimura K, Shah Y, Yang Q, Ward JM and Gonzalez FJ. Spontaneous hepatocarcinogenesis in farnesoid X receptor-null mice. Carcinogenesis 2007; 28: 940-946.
[22] Davis MI, Pragani R, Fox JT, Shen M, Parmar K, Gaudiano EF, Liu L, Tanega C, McGee L, Hall MD, McKnight C, Shinn P, Nelson H, Chattopadhyay D, D'Andrea AD, Auld DS, DeLucas LJ, Li Z, Boxer MB and Simeonov A. Small Molecule Inhibition of the Ubiquitin-specific Protease USP2 Accelerates cyclin D1 degradation and leads to cell cycle arrest in colorectal cancer and mantle cell lymphoma models. J Biol Chem 2016; 291: 24628-24640.
[23] Chen Y, Song X, Valanejad L, Vasilenko A, More V, Qiu X, Chen W, Lai Y, Slitt A, Stoner M, Yan B and Deng R. Bile salt export pump is dysregulated with altered farnesoid X receptor isoform expression in patients with hepatocellular carcinoma. Hepatology 2013; 57: 1530-1541.
[24] Ahsan N, Belmont J, Chen Z, Clifton JG and Salomon AR. Highly reproducible improved label-free quantitative analysis of cellular phosphoproteome by optimization of LC-MS/MS gradient and analytical column construction. J Proteomics 2017; 165: 69-74.
[25] Liu N, Meng Z, Lou G, Zhou W, Wang X, Zhang Y, Zhang L, Liu X, Yen Y, Lai L, Forman BM, Xu Z, Xu R and Huang W. Hepatocarcinogenesis in FXR-/- mice mimics human HCC progression that operates through HNF1alpha regulation

of FXR expression. Mol Endocrinol 2012; 26: 775-785.
[26] Jansen PL. Endogenous bile acids as carcinogens. J Hepatol 2007; 47: 434-435.
[27] Knisely AS, Strautnieks SS, Meier Y, Stieger B, Byrne JA, Portmann BC, Bull LN, Pawlikowska L, Bilezikçi B, Ozçay F, László A, Tiszlavicz L, Moore L, Raftos J, Arnell H, Fischler B, Németh A, Papadogiannakis N, Cielecka-Kuszyk J, Jankowska I, Pawłowska J, Melín-Aldana H, Emerick KM, Whitington PF, Mieli-Vergani G and Thompson RJ. Hepatocellular carcinoma in ten children under five years of age with bile salt export pump deficiency. Hepatology 2006; 44: 478-486.
[28] Sun L, Beggs K, Borude P, Edwards G, Bhushan B, Walesky C, Roy N, Manley MW Jr, Gunewardena S, O'Neil M, Li H and Apte U. Bile acids promote diethylnitrosamine-induced hepatocellular carcinoma via increased inflammatory signaling. Am J Physiol Gastrointest Liver Physiol 2016; 311: G91-G104.
[29] Xie G, Wang X, Huang F, Zhao A, Chen W, Yan J, Zhang Y, Lei S, Ge K, Zheng X, Liu J, Su M, Liu P and Jia W. Dysregulated hepatic bile acids collaboratively promote liver carcinogenesis. Int J Cancer 2016; 139: 1764-1775.
[30] Sodeman T, Bronk SF, Roberts PJ, Miyoshi H and Gores GJ. Bile salts mediate hepatocyte apoptosis by increasing cell surface trafficking of Fas. Am J Physiol Gastrointest Liver Physiol 2000; 278: G992-G999.
[31] Guicciardi ME and Gores GJ. Bile acid-mediated hepatocyte apoptosis and cholestatic liver disease. Dig Liver Dis 2002; 34: 387-392.
[32] Woolbright BL, Dorko K, Antoine DJ, Clarke JI, Gholami P, Li F, Kumer SC, Schmitt TM, Forster J, Fan F, Jenkins RE, Park BK, Hagenbuch B, Olyaee M and Jaeschke H. Bile acid-induced necrosis in primary human hepatocytes and in patients with obstructive cholestasis. Toxicol Appl Pharmacol 2015; 283: 168-177.
[33] Li M, Cai SY and Boyer JL. Mechanisms of bile acid mediated inflammation in the liver. Mol Aspects Med 2017; 56: 45-53.
[34] Priolo C, Tang D, Brahamandan M, Benassi B, Sicinska E, Ogino S, Farsetti A, Porrello A, Finn S, Zimmermann J, Febbo P and Loda M. The isopeptidase USP2a protects human prostate cancer from apoptosis. Cancer Res 2006; 66: 8625-8632.
[35] Boustani MR, Khoshnood RJ, Nikpasand F, Taleshi Z, Ahmadi K, Yahaghi E and Goudarzi PK. Overexpression of ubiquitin-specific protease 2a (USP2a) and nuclear factor erythroid 2-related factor 2 (Nrf2) in human gliomas. J Neurol Sci 2016; 363: 249-252.
[36] Jeong P, Ha YS, Yun SJ, Yoon HY, Freeman MR, Kim J and Kim WJ. Assess the expression of ubiquitin specific protease USP2a for bladder cancer diagnosis. BMC Urol 2015; 15: 1-6.
[37] Meng X, Xiong Z, Xiao W, Yuan C, Wang C, Huang Y, Tong J, Shi J, Chen Z, Liu C, Xie K, Xiong H, Chen K, Yang H and Zhang X. Downregulation of ubiquitin-specific protease 2 possesses prognostic and diagnostic value and promotes the clear cell renal cell carcinoma progression. Ann Transl Med 2020; 8: 319.
[38] Qu Q, Mao Y, Xiao G, Fei X, Wang J, Zhang Y, Liu J, Cheng G, Chen X, Wang J and Shen K. USP2 promotes cell migration and invasion in triple negative breast cancer cell lines. Tumor Biol 2015; 36: 5415-5423.
[39] Xiong B, Huang J, Liu Y, Zou M, Zhao Z, Gong J, Wu X and Qiu C. Ubiquitin-specific protease 2a promotes hepatocellular carcinoma progression via deubiquitination and stabilization of RAB1A. Cell Oncol (Dordr) 2021; 44: 329-343.
[40] Aran D, Camarda R, Odegaard J, Paik H, Oskotsky B, Krings G, Goga A, Sirota M and Butte AJ. Comprehensive analysis of normal adjacent to tumor transcriptomes. Nat Commun 2017; 8: 1077.
[41] Russi S, Calice G, Ruggieri V, Laurino S, La Rocca F, Amendola E, Lapadula C, Compare D, Nardone G, Musto P, De Felice M, Falco G and Zoppoli P. Gastric normal adjacent mucosa versus healthy and cancer tissues: distinctive transcriptomic profiles and biological features. Cancers (Basel) 2019; 11: 1248.
[42] Cameron RG, Imaida K, Tsuda H and Ito N. Promotive effects of steroids and bile acids on hepatocarcinogenesis initiated by diethylnitrosamine. Cancer Res 1982; 42: 2426-2428.
[43] Reglinski K, Keil M, Altendorf S, Waithe D, Eggeling C, Schliebs W and Erdmann R. Peroxisomal import reduces the proapoptotic activity of deubiquitinating enzyme USP2. PLoS One 2015; 10: e0140685.
[44] Principe DR, Doll JA, Bauer J, Jung B, Munshi HG, Bartholin L, Pasche B, Lee C and Grippo PJ. TGF-β: duality of function between tumor prevention and carcinogenesis. J Natl Cancer Inst 2014; 106: djt369.
[45] Seoane J and Gomis RR. TGF-β family signaling in tumor suppression and cancer progression. Cold Spring Harb Perspect Biol 2017; 9: a022277.
[46] Rifaï K, Idrissou M, Penault-Llorca F, Bignon YJ and Bernard-Gallon D. Breaking down the contradictory roles of histone deacetylase SIRT1 in human breast cancer. Cancers (Basel) 2018; 10: 409.
[47] Safe S and Karki K. The Paradoxical roles of orphan nuclear receptor 4A (NR4A) in cancer. Mol Cancer Res 2021; 19: 180-191.
[48] Cebrián MJ, Bauden M, Andersson R, Holdenrieder S and Ansari D. Paradoxical role of

HMGB1 in pancreatic cancer: tumor suppressor or tumor promoter? Anticancer Res 2016; 36: 4381-4389.
[49] Sarvagalla S, Kolapalli SP and Vallabhapurapu S. The two sides of YY1 in cancer: a friend and a foe. Front Oncol 2019; 9: 1230.
[50] Datta N, Chakraborty S, Basu M and Ghosh MK. Tumor suppressors having oncogenic functions: the double agents. Cells 2020; 10: 46.
[51] Lobry C, Oh P and Aifantis I. Oncogenic and tumor suppressor functions of Notch in cancer: it's NOTCH what you think. J Exp Med 2011; 208: 1931-1935.
[52] Chen QY and Costa M. Oncogenic and tumor suppressive roles of special AT-rich sequence-binding protein. J Carcinog 2018; 17: 2.
[53] Ye T, Feng J, Wan X, Xie D and Liu J. Double agent: SPDEF gene with both oncogenic and tumor-suppressor functions in breast cancer. Cancer Manag Res 2020; 12: 3891-3902.
[54] Guerin DJ, Kha CX and Tseng KA. From cell death to regeneration: rebuilding after injury. Front Cell Dev Biol 2021; 9: 655048.
[55] Diwanji N and Bergmann A. Two sides of the same coin - compensatory proliferation in regeneration and cancer. Adv Exp Med Biol 2019; 1167: 65-85.
[56] Fogarty CE and Bergmann A. Killers creating new life: caspases drive apoptosis-induced proliferation in tissue repair and disease. Cell Death Differ 2017; 24: 1390-1400.
[57] Komposch K and Sibilia M. EGFR signaling in liver diseases. Int J Mol Sci 2015; 17: 30.
[58] Hu W, Zheng S, Guo H, Dai B, Ni J, Shi Y, Bian H, Li L, Shen Y, Wu M, Tian Z, Liu G, Hossain MA, Yang H, Wang D, Zhang Q, Yu J, Birnbaumer L, Feng J, Yu D and Yang Y. PLAGL2-EGFR-HIF-1/2α signaling loop promotes HCC progression and Erlotinib insensitivity. Hepatology 2021; 73: 674-691.
[59] Li KS, Zhu XD, Liu HD, Zhang SZ, Li XL, Xiao N, Liu XF, Xu B, Lei M, Zhang YY, Shi WK, Cao MQ, Xu YF, Tang ZY and Sun HC. NT5DC2 promotes tumor cell proliferation by stabilizing EGFR in hepatocellular carcinoma. Cell Death Dis 2020; 11: 335.
[60] Liu Z, Zanata SM, Kim J, Peterson MA, Di Vizio D, Chirieac LR, Pyne S, Agostini M, Freeman MR and Loda M. The ubiquitin-specific protease USP2a prevents endocytosis-mediated EGFR degradation. Oncogene 2013; 32: 1660-1669.
[61] Yu H, Mei XP, Su PF, Jin GZ and Zhou HK. A poor prognosis in human hepatocellular carcinoma is associated with low expression of DPP4. Braz J Med Biol Res 2020; 53: e9114.
[62] Nishina S, Yamauchi A, Kawaguchi T, Kaku K, Goto M, Sasaki K, Hara Y, Tomiyama Y, Kuribayashi F, Torimura T and Hino K. Dipeptidyl Peptidase 4 inhibitors reduce hepatocellular carcinoma by activating lymphocyte chemotaxis in mice. Cell Mol Gastroenterol Hepatol 2018; 7: 115-134.
[63] Kawaguchi T, Nakano D, Koga H and Torimura T. Effects of a DPP4 inhibitor on progression of NASH-related HCC and the p62/ Keap1/Nrf2-pentose phosphate pathway in a mouse model. Liver Cancer 2019; 8: 359-372.
[64] Qin CJ, Zhao LH, Zhou X, Zhang HL, Wen W, Tang L, Zeng M, Wang MD, Fu GB, Huang S, Huang WJ, Yang Y, Bao ZJ, Zhou WP, Wang HY and Yan HX. Inhibition of dipeptidyl peptidase IV prevents high fat diet-induced liver cancer angiogenesis by downregulating chemokine ligand 2. Cancer Lett 2018; 420: 26-37.
[65] Wang G, Bonkovsky HL, de Lemos A and Burczynski FJ. Recent insights into the biological functions of liver fatty acid binding protein 1. J Lipid Res 2015; 56: 2238-2247.
[66] Wang B, Tao X, Huang CZ, Liu JF, Ye YB and Huang AM. Decreased expression of liver-type fatty acid-binding protein is associated with poor prognosis in hepatocellular carcinoma. Hepatogastroenterology 2014; 61: 1321-1326.
[67] Ku CY, Liu YH, Lin HY, Lu SC and Lin JY. Liver fatty acid-binding protein (L-FABP) promotes cellular angiogenesis and migration in hepatocellular carcinoma. Oncotarget 2016; 7: 18229-18246.
[68] Li W, Xiao J, Zhou X, Xu M, Hu C, Xu X, Lu Y, Liu C, Xue S, Nie L, Zhang H, Li Z, Zhang Y, Ji F, Hui L, Tao W, Wei B and Wang H. STK4 regulates TLR pathways and protects against chronic inflammation-related hepatocellular carcinoma. J Clin Invest 2015; 125: 4239-4254.
[69] Zhou D, Conrad C, Xia F, Park JS, Payer B, Yin Y, Lauwers GY, Thasler W, Lee JT, Avruch J and Bardeesy N. Mst1 and Mst2 maintain hepatocyte quiescence and suppress hepatocellular carcinoma development through inactivation of the Yap1 oncogene. Cancer Cell 2009; 16: 425-438.
[70] Avruch J, Zhou D, Fitamant J and Bardeesy N. Mst1/2 signalling to Yap: gatekeeper for liver size and tumour development. Br J Cancer 2011; 104: 24-32.
[71] Engel E, Viargues P, Mortier M, Taillebourg E, Couté Y, Thevenon D and Fauvarque MO. Identifying USPs regulating immune signals in Drosophila: USP2 deubiquitinates Imd and promotes its degradation by interacting with the proteasome. Cell Commun Signal 2014; 12: 41.