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Original Article

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

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Abstract: Ubiquitin specific peptidase 2a (USP2a) plays critical roles in protein degradation and other cellular activities. Currently, our understanding on USP2a dysregulation in subjects with hepatocellular carcinoma (HCC) and its roles in HCC pathogenesis is limited. In this study, we found that USP2a mRNA and protein levels were significantly upregulated in HCC tumors from both human and mice. USP2a overexpression in HepG2 and Huh 7 cells significantly increased cell proliferation while inhibition of USP2a activity by chemical inhibitor or stable knockout of USP2 by CRISPR markedly reduced cell proliferation. In addition, USP2a overexpression significantly augmented the resistance while knockout of USP2a markedly increased the susceptibility of HepG2 cells to bile acid-induced apoptosis and necrosis. Consistent with the oncogenic activities detected *in vitro*, overexpression of USP2a promoted *de novo* HCC development in mice with significantly increased tumor occurrence rates, tumor sizes and liver/body ratios. Further investigations with unbiased co-immunoprecipitation (Co-IP)-coupled proteomic analysis and Western blot identified novel USP2a target proteins involved in cell proliferation, apoptosis, and tumorigenesis. Analysis of those USP2a target proteins revealed that USP2a's oncogenic activities are mediated through multiple pathways, including modulating protein folding and assembling through regulating protein chaperones/co-chaperones HSPA1A, DNAJA1 and TCP1, promoting DNA replication and transcription through regulating RUVBL1, PCNA and TARDBP, and altering mitochondrial apoptotic pathway through regulating VDAC2. Indeed, those newly identified USP2a target proteins were markedly dysregulated in HCC tumors. In summary, USP2a was upregulated in HCC subjects and acted as an oncogene in the pathogenesis of HCC through multiple downstream pathways. The findings provided molecular and pathogenesis bases for developing interventions to treat HCC by targeting USP2a or its downstream pathways.

Keywords: USP2a, HCC, apoptosis, 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]. The etiology and pathogenesis of HCC are complex and imposes significant challenges to understanding the underlying mechanisms as well as development of effective therapeutic interventions [1, 2]. Viral infection, alcohol usage, and non-alcohol liver fat diseases (NAFLD) are the major causes for chronic liver injury and eventually for HCC development [3]. At present, there are limited options in treating HCC patients [4]. Liver resection, ablation, or liver transplantation are the only curative options for HCC patients at early stage. However, a major proportion of HCC patients who are diagnosed with intermediate and advanced stages have limited treatment options [5]. Therefore, there is urgent need to develop therapies for HCC.

Ubiquitination is one of the most common post-translational protein modifications, playing critical roles in protein degradation through the proteasomal pathway, protein localization and activities as well as protein-protein interaction [6]. On the other hand, ubiquitination can be reversed by deubiquitinating enzymes [7]. Therefore, maintenance of the ubiquitination and deubiquitination homeostasis is crucial for a plethora of physiological processes, including embryonic development, cell survival, differentiation, and cell cycle controls. Dysregulation of

the ubiquitination/deubiquitination has been implicated in the pathogenesis of multiple cancers including HCC [7, 8]. As a member of deubiquitinating enzymes, ubiquitin specific peptidase 2 (USP2) removes ubiquitin from ubiquitinated target proteins, thus preventing proteasomal degradation and stabilizing the target proteins [9, 10]. We have previously reported that the liver abundantly expresses two USP2 isoforms USP2a and USP2b, and USP2b was dysregulated in HCC subjects exhibiting tumor-suppressing and tumor-promoting activities in a context-dependent manner [11].

USP2a has been implicated in various physiological and pathophysiological conditions, including energy metabolism, ion channel homeostasis and especially tumorigenesis [12]. Dependent on the type of cancers, USP2a exhibited both tumor-promoting and tumor-suppressing activities [13-19]. USP2a promoted bladder cancer progression by stabilizing the cell cycle regulator cyclin A1 [13]. In prostate cancer, USP2a exhibited oncogenic properties by enhancing Myc activation and stabilizing fatty acid synthase (FASN) [14, 15]. It was also reported that USP2a played important roles in the pathogenesis of glioma through regulating FASN, erythroid 2-related factor 2 (Nrf2) and mouse double minute 4 (MDM4)/p53 pathways [16-18]. However, in clear cell renal cell carcinoma, USP2a was markedly down-regulated and acted as a tumor-suppressor [19]. For HCC, an early study showed that USP2a regulated FASN, which may contribute to the pathogenesis of HCC [20]. Another study reported that USP2a increased hepatoma cell growth *in vitro* and *in vivo* in cell line-grafted immunocompromised nude mouse models through deubiquitinating and stabilizing Ras-related protein Rab-1A (RAB1A) [21]. Therefore, our current understanding of USP2a's roles in the pathogenesis of HCC is limited or incomplete, especially in USP2a-mediated regulation on cell death including apoptosis and necrosis, *de novo* HCC development *in vivo* and its downstream pathways.

In this study, we found that in contrast to USP2b [11], USP2a expression was significantly up-regulated in HCC subjects. USP2a exhibited oncogenic activities by promoting cell proliferation as well as exerting potent anti-apoptotic and anti-necrotic activities. More importantly, USP2a significantly promoted *de novo* HCC development *in vivo* in a chemically induced HCC mouse model with increased tumor occurrence and size. Unbiased coimmunoprecipitated (Co-IP)-coupled proteomic analysis and Western blot identified novel USP2a target proteins directly involved in cell proliferation, apoptosis, and tumorigenesis. Analysis of those USP2a target proteins revealed that USP2a's oncogenic activities are mediated through multiple pathways, including modulating protein folding and assembling by regulating protein chaperones/co-chaperones heat shock protein family A (Hsp70) member 1A (HSPA1A), DnaJ heat shock protein family (Hsp40) member A1 (DNAJA1) and T-Complex 1 (TCP1), promoting DNA replication and transcription by regulating RuvB like AAA ATPase 1 (RUVBL1), TAR DNA binding protein (TARDBP) and proliferating cell nuclear antigen (PCNA), and altering mitochondrial apoptotic pathway by regulating mitochondrial voltage-dependent anion channel 2 (VDAC2). Indeed, those newly identified USP2a target proteins were markedly dysregulated in the HCC tumor. The findings thus concluded that USP2a exhibited oncogenic or tumor-promoting activities *in vitro* and *in vivo* through regulating multiple downstream pathways. The findings, therefore, provided molecular and pathogenesis bases for developing therapies for HCC through targeting USP2a or its downstream pathways.

Materials and methods

Cells and constructs

Human hepatoma cell line HepG2 (cat#: HB-8065) and Huh 7 (cat#: PTA-4583) cells were obtained from American Type Culture Collection (Manassas, VA, USA). USP2 knockout (USP2-KO) cells were generated using parental HepG2 cells with pLentiCRISPR v2 USP2 sgRNA as described [11]. Two independent USP2-KO cell clones, USP2-KO (3A) and USP2-KO (3E) were used in the study. The expression construct for human USP2a was custom-made with codon optimization for human cell expression and expressed in the pcDNA3.1(+) vector (Gene Universal, Newark, DE, USA).

Human liver samples

Fifteen normal human liver and 21 HCC tumor (HCC-T) with 8 paired HCC adjacent non-tumor

(HCC-NT) tissues were obtained through the Cooperative Human Tissue Network (Rockville, MD, USA). The study was approved by the Institutional Review Board at the University of Rhode Island (URI). The characteristics of the HCC patients were reported previously [22].

Mouse liver samples

Farnesoid X receptor knockout (FXR-KO) mice were obtained from the Jackson Laboratory (stock no: 007214, Bar Harbor, Maine, USA). Mice were fed a regular chow diet. A total of 26 HCC-T and HCC-NT tissue samples were collected from 12 female (F) and 14 male (M) FXR-KO mice at the ages of 14-18 months when HCC spontaneously developed [23]. Paired HCC-T and HCC-NT tissues 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 of URI.

USP2a's effects on diethylnitrosamine (DEN)-induced de novo HCC development in mice

C57BL/6 mice at the age of 2 weeks were peritoneally injected with a single dose of DEN (50 mg/kg) (MilliporeSigma, Burlington, MA, USA). At the age of 3-4 months, the mice were hydrodynamically injected with USP2a expression construct or pcDNA vector plasmid DNA at a dose of 0.5 μg/g via tail vein. The mice were fed with regular chow diet during the entire study. The mice were euthanized at the age of 13-14 months. Body and liver weights were recorded. Visible tumors in the liver were counted and the tumor sizes were measured.

Cell proliferation assays

HepG2 or Huh 7 cells were transfected with USP2a or pcDNA vector with or without treatment of USP2 specific inhibitor ML364 (2 μM) (MedChem Express, Princeton, NJ, USA). Cell viability was detected by the MTS assays (cat#: G9242, Promega, Madison, WI, USA). Cell proliferation assays were also carried out by viable cell counting.

Apoptosis and necrosis assays

HepG2 and USP2-KO cells were treated with various concentrations of chenodeoxycholic acid (CDCA) or vehicle for various time points, followed by detection of the apoptotic and necrotic activities with the RealTime-Glo Annexin V Apoptosis and Necrosis Assays (cat#: JA1012, Promega, Madison, WI, USA).

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 [22]. TaqMan PCR probes including human USP2a (Hs00374431_m1), human glyceraldehyde 3-phosphate dehydrogenase (GAPDH) (4352665), mouse Usp2a (ARCE37A) and mouse Gapdh (4352932E) were purchased from Thermo Fisher Scientific (Franklin, MA, USA). Transcript levels of USP2a were normalized against GAPDH levels.

Coimmunoprecipitation (Co-IP) assays

USP2-KO (3A) cells were transfected with c-Myc tagged USP2a or empty vector to overexpress USP2a protein. After the transfected cells were lysed with RIPA buffer, cell lysates of 500 μg total protein underwent Co-IP with the Pierce c-Myc-Tag IP/Co-IP Kit (cat#: 23620, from ThermoFisher Scientific, Franklin, MA, USA) following the procedure provided by the protocol.

Western blot

Lysates from human or mouse liver tissues, USP2-KO cells or co-immunoprecipitated complexes were used for Western blot as previously described [22]. Goat anti-human USP2a antibodies (AF5804) were purchased from R&D Systems (NE Minneapolis, MN, USA) or custom-made rabbit anti-human USP2a-specific antibodies. Mouse anti-human TARDBP (sc-376532), HSPA1A (sc-24), DNAJA1 (sc-59554), RUVBL1 (sc-393905), PCNA (sc-56), rat anti-human TCP1 (sc-53454), GAPDH (sc-365062), and secondary antibodies goat-anti-mouse IgG-HRP (sc-2005) and mouse anti-rabbit IgG HRP (sc-2357) were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Rabbit anti-human VDAC2 (PA5-28106) and secondary antibodies rabbit anti-goat antibodies (81-1620) and goat anti-mouse IgG HRP (G21040) were purchased from ThermoFisher Scientific (Franklin, MA, USA).

Co-IP coupled proteome analysis

USP2-KO (3A) cells were transfected with C-Myc tagged USP2a or empty vector to overexpress

USP2a protein. After the transfected cells were lysed with RIPA buffer, cell lysates of 500 μg total protein underwent Co-IP with the Pierce c-Myc-Tag IP/Co-IP Kit (cat#: 88844, Thermo Fisher Scientific, Franklin, MA, USA) following the procedure provided by the protocol. Proteins were eluted off from the agarose beads, precipitated and digested with the iST kit (PreOmics GmbH, Martinsried, Germany) per manufacturers protocol. The resulting peptides were reconstituted in 0.1% TFA containing iRT peptides (Biognosys, Schlieren, Switzerland). Four independent Co-IP protein samples from USP2a or empty vector were subjected to MS/MS proteomic analysis. Samples were analyzed on an Exploris 480 mass spectrometer (Thermo Fisher Scientific, San Jose, CA, USA) coupled with an Ultimate 3000 nano UPLC system and an EasySpray source using data dependent acquisition. MS/MS raw files were searched using MaxQuant (v. 1.6.1.0) against a human protein sequence database. Scaffold (version Scaffold_5.1.0, Proteome Software Inc., Portland, OR, USA) was used to validate MS/MS based peptide and protein identifications. Kyoto encyclopedia of genes and genomes (KEGG) pathways and ShinyGo gene ontology enrichment analyses 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

USP2a expression was significantly upregulated in HCC subjects

To determine whether USP2a expression was altered in HCC, the expression levels of USP2a mRNA and protein in normal human liver, HCC-NT and HCC-T tissues were evaluated by real-time PCR and Western blot. As shown in **Figure 1A**, USP2a mRNA levels were significantly upregulated in HCC-NT ($P < 0.05$) and HCC-T ($n=21$, $P < 0.05$) tissues when compared to the normal liver tissues. Consistent with the mRNA levels, USP2a protein expression was also significantly upregulated in HCC-T ($P < 0.05$) (**Figure 1B**) when compared to the normal liver tissues. Compared with HCC-NT, USP2a expression exhibited an increasing trend in HCC-T tissues (**Figure 1A** and **1B**) without reaching statistical significance. However, when we looked at the individual pairs of HCC-NT and HCC-T tissues, USP2a expression was markedly upregulated in five out of eight pairs (62.5%) with noticeably reduced expression in 2 pairs (25%) and no difference in one pair (12.5%) (**Figure 1D**). Consistent with the findings from human HCC subjects, Usp2a mRNA and protein expression exhibited increasing trends in HCC-T compared to HCC-NT tissues from mouse HCC subjects (**Figure 1D** and **1F**). At the individual pair level, USP2 mRNA expressions were markedly increased in 11 out of 26 pairs (42.3%) with a reduction in 7 pairs (26.9%) and no significant differences in 8 pairs (30.8%) (**Figure 1E**). Similarly, Usp2a protein abundances were increased in 66.7% of the paired HCC-T tissues with a reduced expression in 33.3% of the pairs when compared to the corresponding HCC-NT tissues (**Figure 1G**), which is largely correlated with mRNA expression levels.

Taken together, the results demonstrated that USP2a expression in HCC-T tissues was significantly upregulated at both mRNA and protein levels when compared to normal liver tissues while exhibiting an increasing trend with markedly increased expression in a large portion of the samples when compared to the HCC-NT tissues from both human and mouse.

USP2a promoted cell proliferation

To determine if USP2a is involved in regulating cell proliferation, a series of cell proliferation assays were carried out. Overexpression of USP2a in HepG2 cells significantly increased cell growth ($P < 0.01$ or $P < 0.001$) when compared to the pcDNA vector-transfected cells (**Figure 2A**). Similarly, overexpression of USP2a markedly promoted cell proliferation in Huh 7 cells ($P < 0.001$) (**Figure 2B**). Consistently, inhibition of USP2 activities with a selective USP2 inhibitor ML364 significantly reduced cell proliferation ($P < 0.05$) (**Figure 2C**).

To further confirm the roles of USP2a in cell proliferation, stable USP2-KO cells were generated using CRISPR (6) and its effects on cell growth

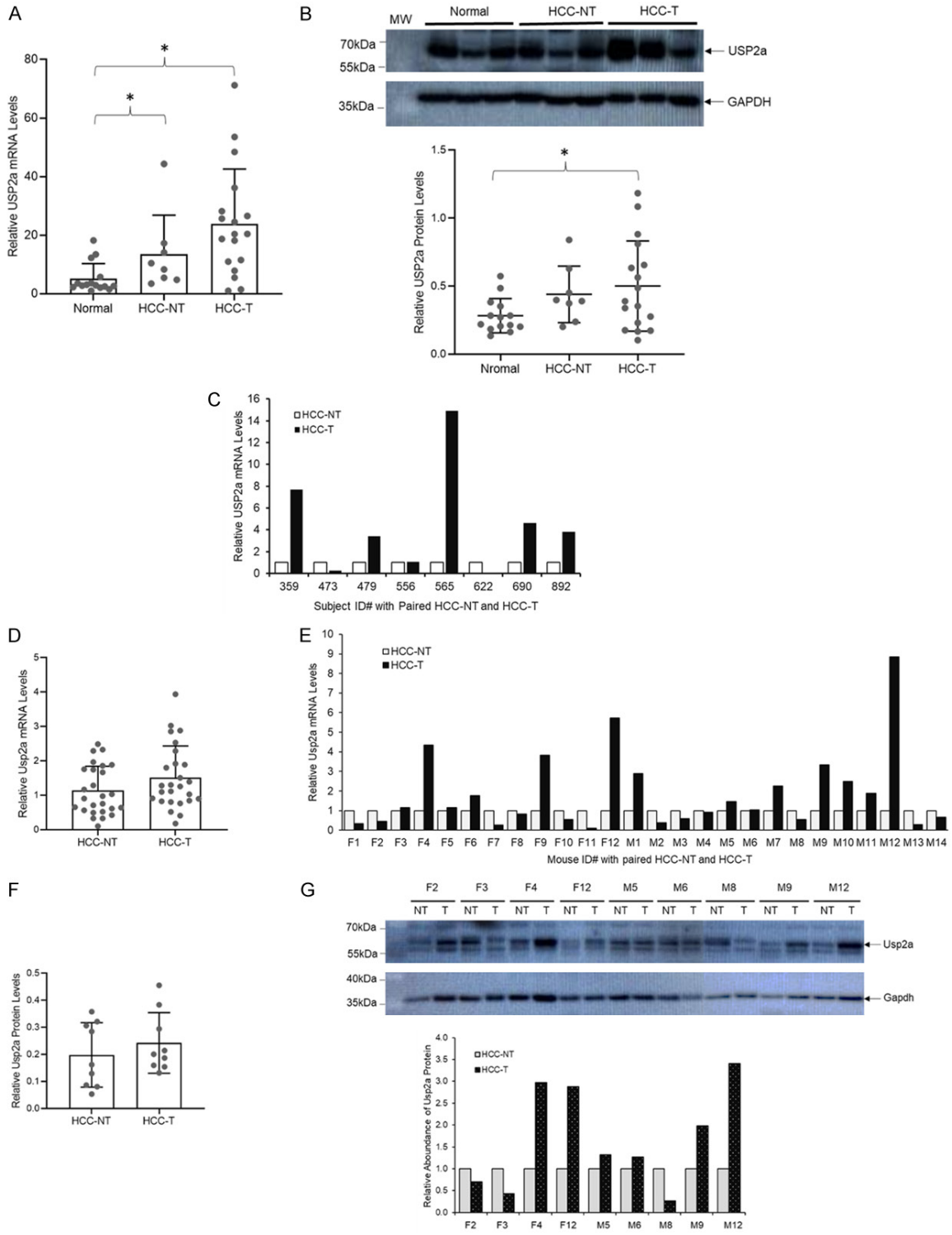


Figure 1. USP2a expression was dysregulated in HCC subjects. (A) The expression levels of USP2a mRNA in human normal liver (n=12), HCC-NT (n=8) and HCC-T (n=21) were detected by real-time PCR. (B) USP2a protein levels in human normal liver (n=14), HCC-NT (n=8) and HCC-T (n=17) were detected and quantified by Western blot. The results from three representative tissue samples from each of the three groups (normal, HCC-NT and HCC-T) were presented in the upper panel. (C) The expression levels of USP2a mRNA in individual paired HCC-T and HCC-NT tissues (n=8). (D) The expression levels of Usp2a mRNA in mouse HCC-NT (n=26) and HCC-T tissues (n=26) collected from 12 female (F1 to F12) and 14 male (M1 to M14) mice were detected by real-time PCR as groups and (E) individual pairs. (F) Usp2a protein levels in mouse paired HCC-NT and HCC-T tissues (n=9) were detected and quantified by Western blot as groups and (G) individual pairs. The experiments with real-time were performed twice with two repeats for each sample while Western blots were performed once or twice depending on the quality of the results. *$P < 0.05$ with one-way ANOVA, followed by Tukey post-hoc test for multiple comparisons.

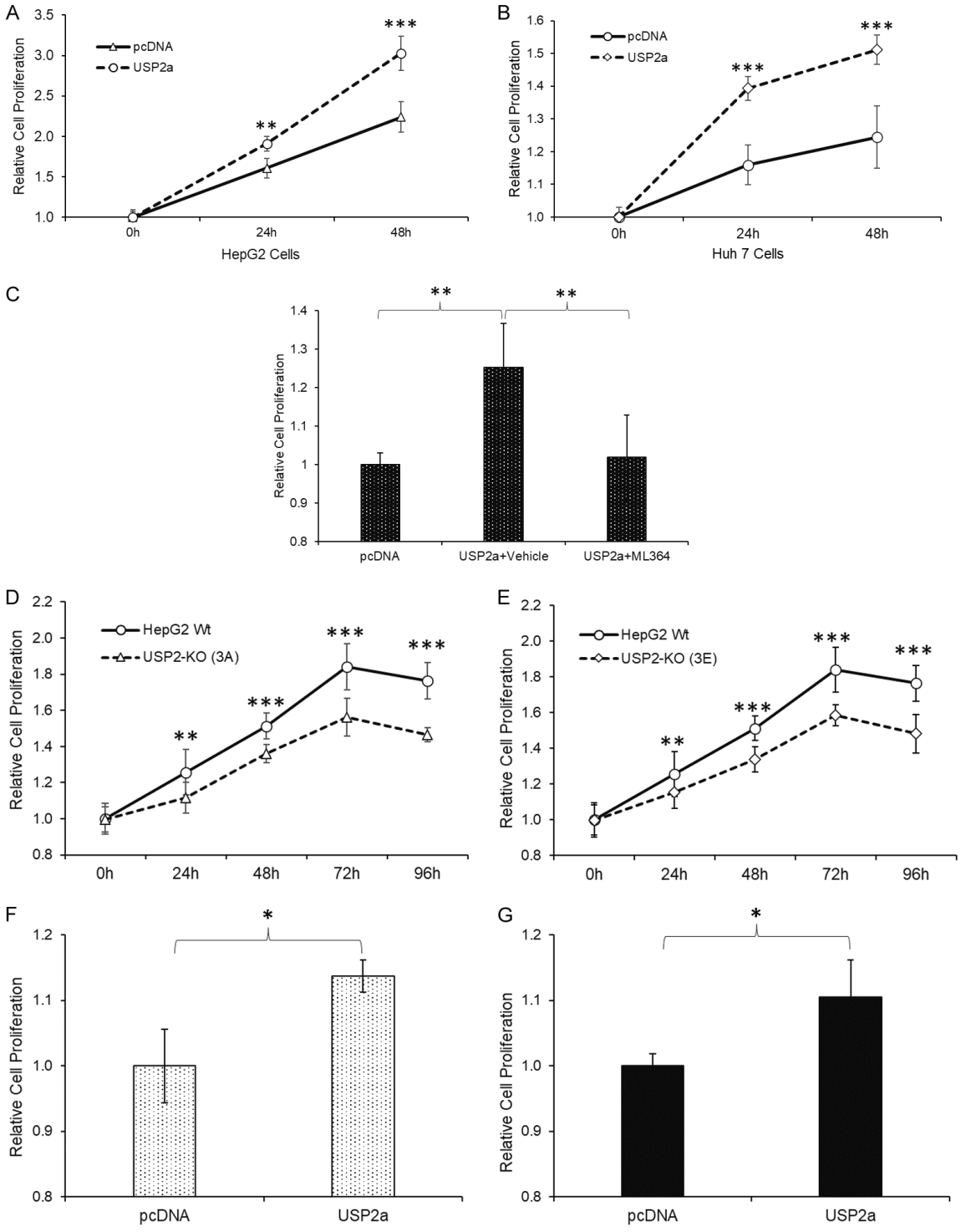


Figure 2. USP2a promoted cell proliferation. (A) HepG2 and (B) Huh 7 cells were transfected with USP2a or pcDNA vector as a control, followed by monitoring cell growth for a period of 48 h by the MTS assays. (C) HepG2 cells were transfected with USP2a or pcDNA vector in the presence or absence of USP2 inhibitor ML364 (2 μM) for 48 h, followed by detection of cell proliferation by MTS assays. (D) Relative proliferation of parental HepG2 wt and two independent USP2 knockout cell clones, USP2-KO (3A) and (E) USP2-KO (3E), over a period of 96 h. (F) USP2-KO (3A) and (G) USP2-KO (3E) cells were transfected with USP2a or pcDNA vector, followed by detection of the cell proliferation at 48 h post-transfection. All the experiments were performed at least twice with 4-6 repeats for each treatment or condition. $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$ in students' t-test for pair-wise comparison or one-way ANOVA, followed by Tukey post-hoc test for multiple comparisons.

were evaluated together with parental HepG2 wt cells. Two independent cell clones, USP2-KO (3A) and USP2-KO (3E), were characterized. As shown in **Figure 2D** and **2E**, the cell proliferation rates of USP2-KO (3A) and USP2-KO (3E) were significantly reduced compared to the parental HepG2 cells ($P < 0.01$ or $P < 0.001$). Consistently, overexpression of USP2a in both USP2-KO cells significantly increased cell growth ($P < 0.05$) (**Figure 2F** and **2G**). Taken together, the results firmly established that USP2a promoted cell proliferation.

Knockout of USP2 made the USP2-KO cells more susceptible to bile acid-induced apoptosis and necrosis

HCC development is often preceded by various liver injuries with a concurrent elevation of hepatic bile acids. Elevated bile acids induce cell death and are directly linked to the development and progression of HCC [24-27]. Bile acids-induced cell death can be categorized into two types, apoptosis [28, 29] and necrosis [30, 31]. Therefore, we chose bile acid chenodeoxycholic acid (CDCA) as an apoptosis and necrosis inducer to investigate USP2a's roles in hepatocyte apoptosis and necrosis, which is clinically relevant to HCC development *in vivo*. To determine the effects of USP2 knockout on bile acid-induced apoptosis, the apoptotic activities of USP2-KO and HepG2 wt cells in the presence of increasing concentrations of CDCA were detected. As shown in **Figure 3A**, when CDCA concentrations increased to 300 μM or above, USP2-KO cells exhibited significantly higher apoptotic activities than HepG2 wt cells ($P < 0.05$ or $P < 0.01$), indicating that loss of USP2 makes USP2-KO cells more susceptible to bile acids-induced apoptosis. A time course study revealed that apoptosis induced by CDCA (500 μM) peaked at 2-4 hours post-treatment for both HepG2 and USP2-KO cells at distinctly different levels (**Figure 3B**).

To determine whether USP2 knockout has any effects on bile acid-induced necrosis, USP2-KO and HepG2 wt cells were treated with increasing concentrations of CDCA, followed by the detection of necrotic activities. As shown in **Figure 3C**, significantly higher necrotic activities were detected in USP2-KO than in HepG2 wt cells when the CDCA concentrations reaches 400 μM or above ($P < 0.01$). Time course study showed that necrotic activities were detected as early as 2 hours and peaked at 8 to 12 hours post-treatment for USP2-KO cells while necrotic activities were detected at 4 hours and peaked at 12 to 24 hours post-treatment for HepG2 wt cells (**Figure 3D**). USP2-KO cells had significantly higher necrotic activities than HepG2 wt cells from 2 to 12 hours post-treatment ($P < 0.001$ or $P < 0.01$) (**Figure 3D**). After 12 hours, the necrotic activities of USP2-KO cells quickly dropped since the majority of cells in the wells were already dead at 12 hours post-treatment. On the other hand, additional necrotic activities were detected in HepG2 wt cells at 24 and 48 h post-treatment (**Figure 3D**). Taken together, the results demonstrated that USP2 knockout significantly increased the susceptibility of the USP2-KO cells to bile acid-induced apoptosis and necrosis when compared to the HepG2 wt cells.

Expression of USP2a markedly increased the resistance of the USP2-KO cells to bile acid-induced apoptosis and necrosis

To determine whether expression of USP2a in USP2-KO cells can restore their resistance to bile acid-induced apoptosis and necrosis, USP2a was transfected into USP2-KO cells in the presence of increasing concentrations of CDCA followed by detection of apoptotic and necrotic activities. As shown in **Figure 3E** and **3F**, expression of USP2a in USP2-KO cells markedly increased the resistance of the transfected cells to CDCA-induced apoptosis ($P < 0.001$) and necrosis ($P < 0.001$), respectively. Taken together, the data from USP2a knockout and forced expression studies firmly established that USP2a had potent anti-apoptotic and anti-necrotic activities in hepatocytes.

USP2a promoted de novo HCC development in vivo in mice

The results from our *in vitro* studies revealed that USP2a acted as an oncogene by promoting cell proliferation and exhibiting potent anti-apoptotic and anti-necrotic activities. To confirm such oncogenic activities *in vivo*, the effects of USP2a overexpression on *de novo* HCC development was investigated in DEN-induced HCC mouse model. As shown in **Figure 4A** and **4B**, the percentages of mice developing visible HCC tumors were significantly increased from 57.1% in mice injected with pcDNA to 100% in mice injected with USP2a ($P < 0.05$).

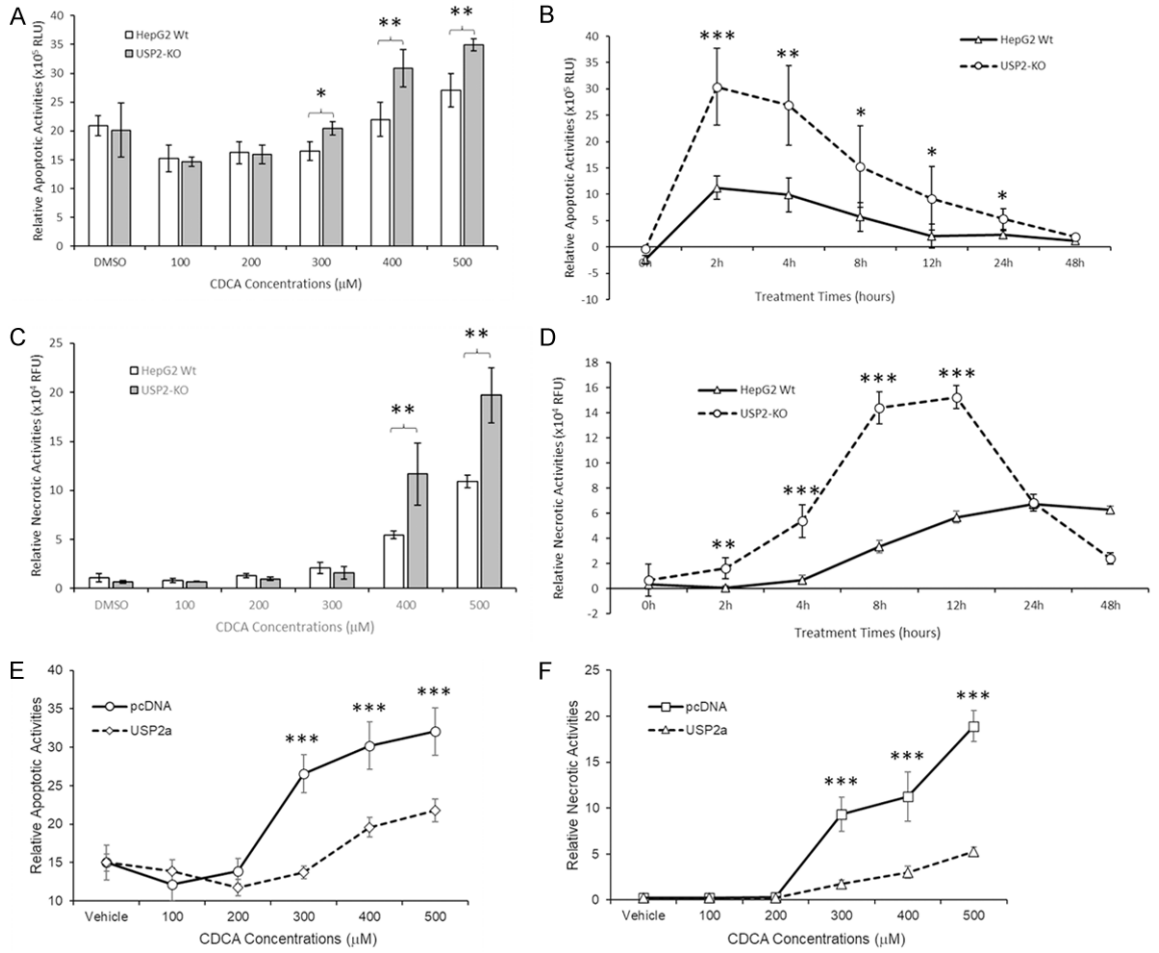


Figure 3. USP2a exhibited anti-apoptotic and anti-necrotic activities. A. HepG2 wt and USP2-KO cells were treated with increasing concentrations of CDCA or vehicle DMSO for 4 h, followed by detection of apoptotic activities with the RealTime-Glo Annexin V Apoptosis assays. B. HepG2 wt and USP2-KO cells were treated with CDCA (500 μM) and apoptotic activities were detected at various time points over a period of 48 h. C. HepG2 wt and USP2-KO cells were treated with increasing concentrations of CDCA or vehicle DMSO for 8 h, followed by detection of necrotic activities with the RealTime-Glo Necrosis assays. D. HepG2 wt and USP2-KO cells were treated with CDCA (500 μM) and necrotic activities were detected at various time points over a period of 48 h. E. USP2-KO cells were transfected with USP2a or pcDNA vector, followed by treatment of the transfected cells with increasing concentrations of CDCA for 4 h. Apoptotic activities were then quantified. F. USP2-KO cells were transfected with USP2a or pcDNA vector, followed by treatment of the transfected cells with increasing concentrations of CDCA for 8 h. Necrotic activities were then quantified. All the experiments were performed at least twice with 4-6 repeats for each treatment or condition. $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$ with students' t-test for pair-wise comparison.

Mice injected with USP2a developed more HCC tumors with increased tumor sizes when compared to the mice injected with vector pcDNA. The average number of HCC tumors per animal increased from 1.9±2.7 in mice injected with pcDNA to 5.7±5.3 in mice injected with USP2a without reaching a statistical significance due to large variations in tumor numbers per mouse within the groups (**Figure 4C**). The average total tumor volumes per animal were significantly increased from 0.068±0.134 mm^3 in mice injected with pcDNA to 1.962±2.286 mm^3 in mice injected with USP2a ($P < 0.05$) (**Figure 4D**).

The average body weights were slightly decreased in USP2a-treated mice (28.1±5.3 g) when compared to the mice treated with pcDNA (30.3±3.9 g) without reaching a statistical significance (P=0.36) (**Figure 4E**). The average liver weights were increased from 1.2±0.3 g in pcDNA-treated mice to 2.6±1.8 g in mice injected with USP2a (**Figure 4F**). Consistently, the liver/body weight ratios were significantly ele-

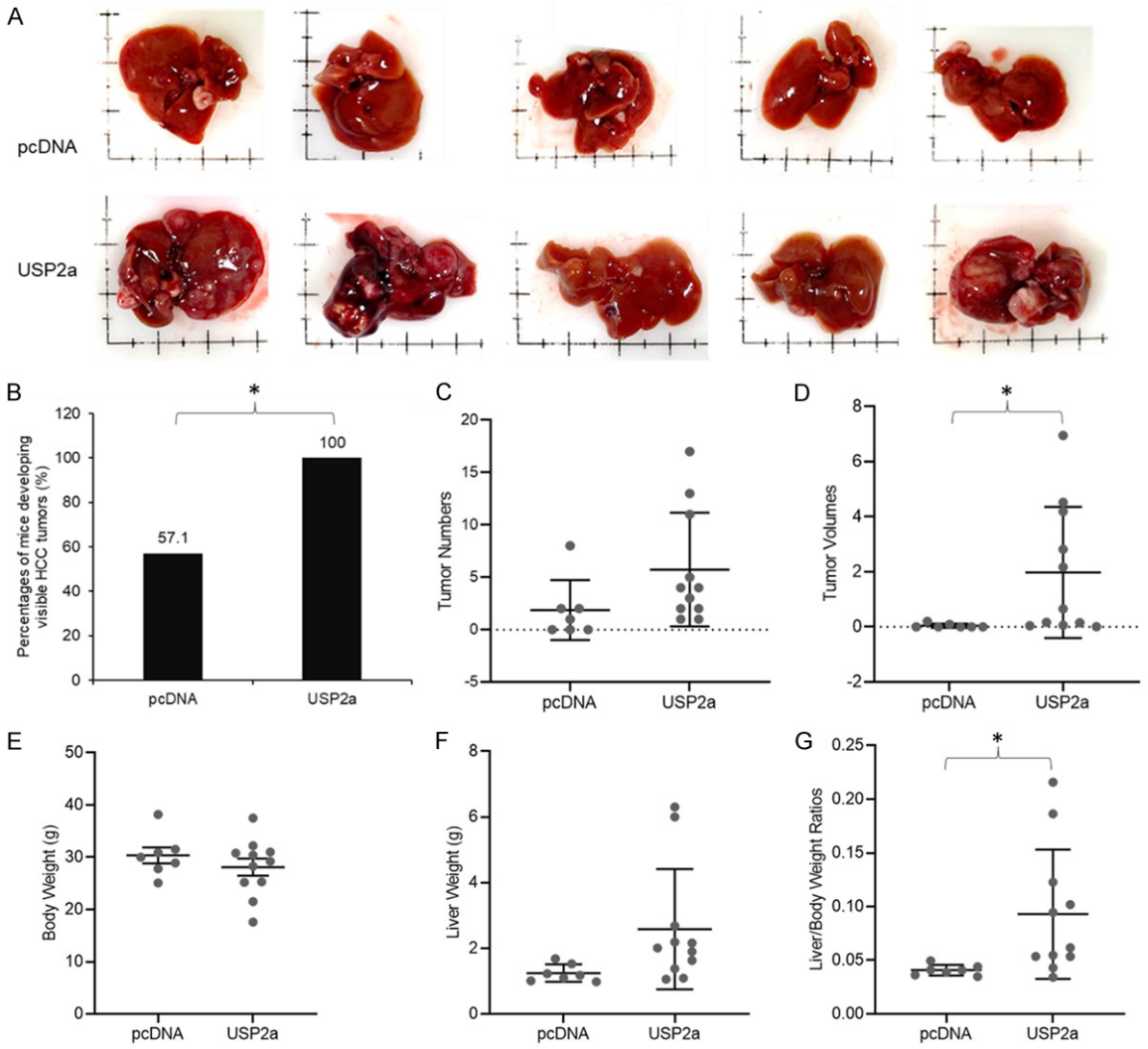
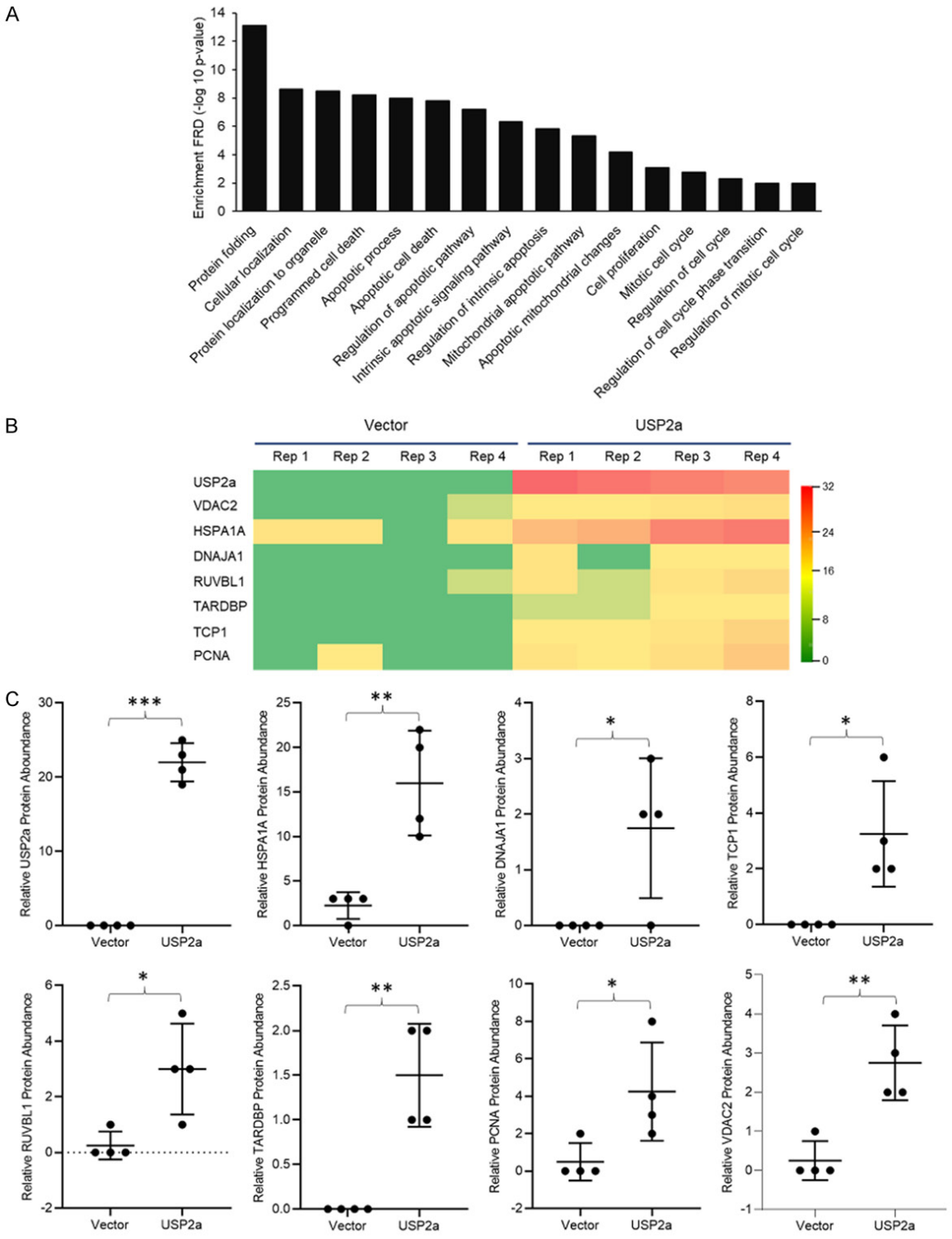


Figure 4. USP2a promoted HCC development *in vivo* in mice. (A) C57BL/6 mice at the age of 2-3 weeks were peritoneally injected with a single dose of DEN (50 mg/kg). At the age of 3-4 months, the mice were hydrodynamically injected with USP2a (n=7) or pcDNA vector (n=11) plasmid DNA at a dose of 0.5 μg/g via tail vein. The mice were fed with regular chow diet during the entire study. The mice were euthanized at the age of 13-14 months. The liver tissues of mice treated with USP2a or pcDNA were presented. (B) The percentages of mice developed visible tumors in mice treated with USP2a or pcDNA. (C) Total visible tumor numbers and (D) volumes of individual mice treated with USP2a or pcDNA. (E) Body weight, (F) liver weight and (G) liver/body weight ratios of mice treated with USP2a or pcDNA. This long-term mouse study was performed once. *$P < 0.05$ with students' t-test for pair-wise comparison.

vated from 0.041±0.005 in mice treated with pcDNA to 0.093±0.059 in mice injected with USP2a ($P < 0.05$) (**Figure 4G**). Taken together, the results demonstrated that USP2a acted as an oncogene promoting *de novo* HCC tumor development in mice.

Identification of USP2a-regulated downstream target proteins

As a deubiquitinating enzyme, USP2a physically interacts with its target proteins or substrates. To identify the downstream target proteins of USP2a that mediate its oncogenic activities, we performed an unbiased Co-IP coupled MS/MS proteomic analysis. A total of 44 proteins were identified as potential USP2a substrates or targets with 23 proteins being detected in USP2a-transfected cells only while 21 proteins were significantly elevated in USP2a-transfected cells compared to the cells transfected with vector control. Gene enrichment and pathway analysis of these proteins revealed several cellular functional pathways associated with those proteins (**Figure 5A**). Protein folding and localization are the top two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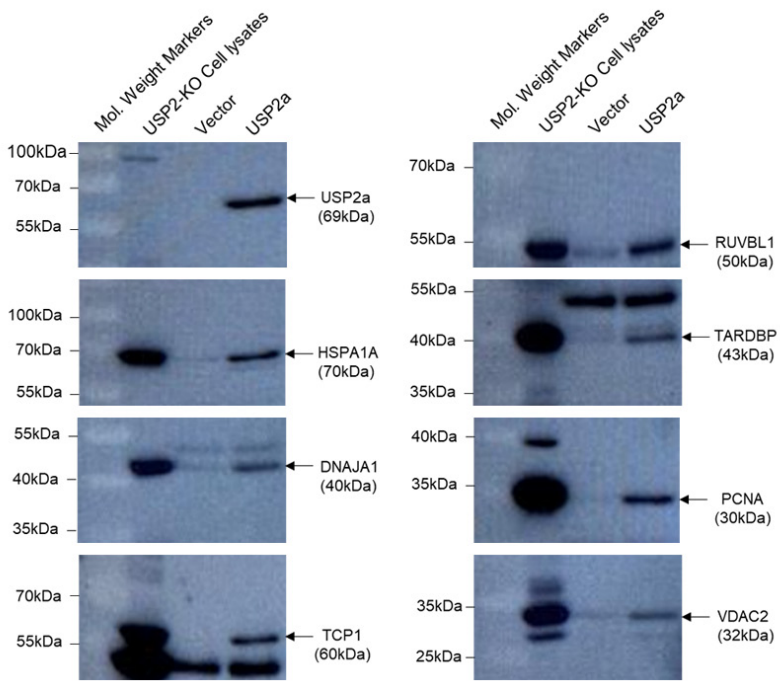


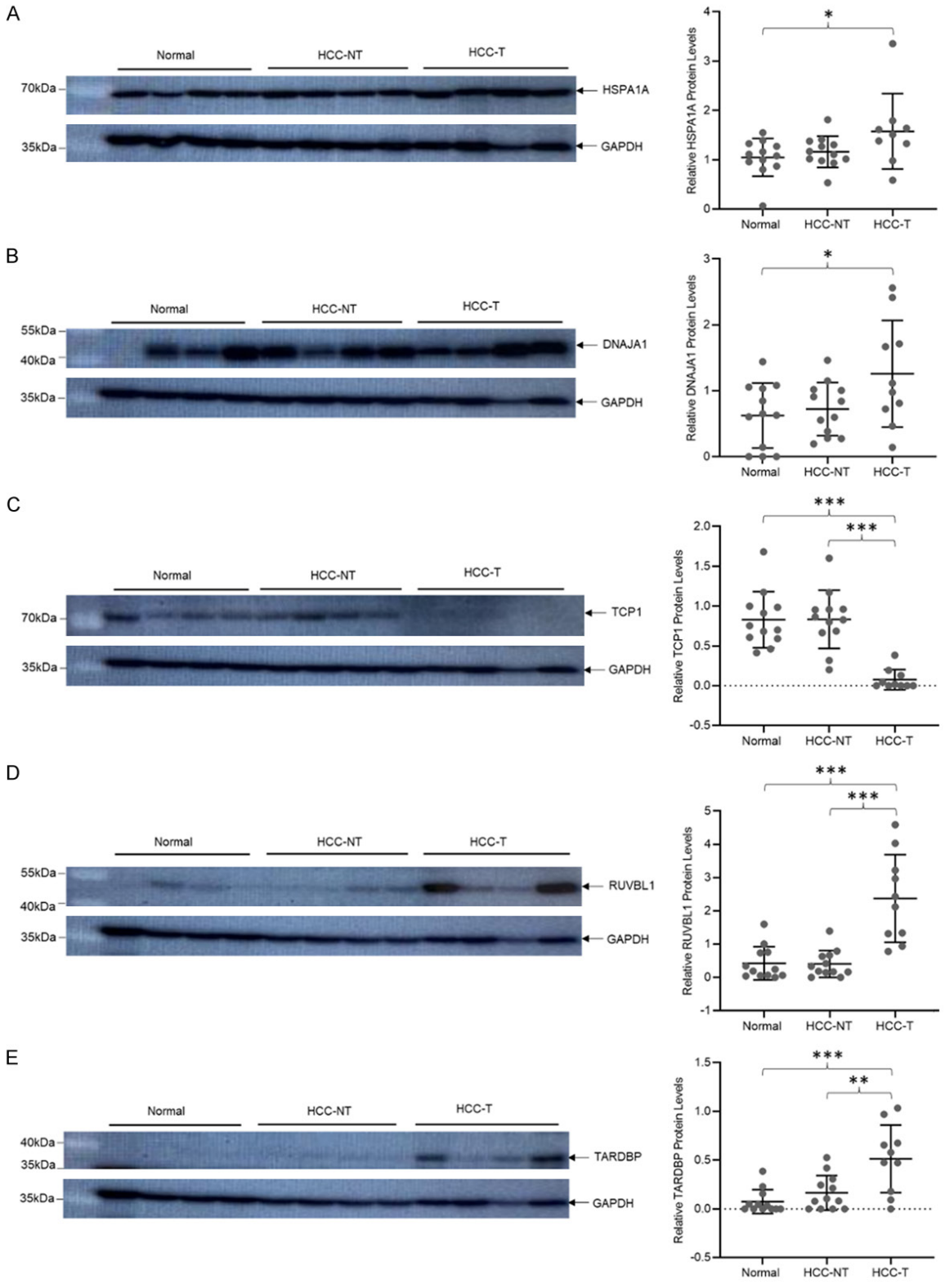
Figure 5. Identification of USP2a target proteins by Co-IP coupled MS/MS proteomic analysis and Co-IP coupled Western blot confirmation. (A) USP2-KO cells were transfected with USP2a or pcDNA vector, followed by Co-IP to pull down USP2a-associated protein complexes, which were subjected to MS/MS proteomic analysis to identify USP2a target proteins. Four replicates for each treatment were subjected to proteomic analysis. The pathway/cellular process enrichment analysis of the identified proteins was carried out with KEGG and ShinyGo. (B) The heat map analysis and (C) relative abundances of USP2a and its seven target proteins in the immunoprecipitated protein complexes detected by MS/MS and (D) confirmed by Western blot. The Co-IP assays were performed four times while the MS/MS proteomic detection was performed once with four repeats in each group. $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$ with students' t-test for pair-wise comparison.

most enriched pathways, indicating that USP2a plays important roles in regulating proteins involved in protein folding and localization. The target proteins are also significantly enriched in regulating programmed cell death/apoptosis, cell proliferation and cycle control (**Figure 5A**). Notably, we identified seven novel target proteins that were abundantly associated with USP2a in the Co-IPed USP2a protein complexes (**Figure 5B** and **5C**) and are directly involved in cell proliferation/growth, cell death/apoptosis and the pathogenesis of various cancers. Among the seven target proteins, three are chaperones or co-chaperones including HSPA1A, DNAJA1 and TCP1. The other three target proteins directly participate in DNA replication and transcription including RUVBL1, TARDBP and PCNA. The last target protein is VDAC2, which is a critical player in the mitochondrial apoptotic pathway.

To confirm the results from MS/MS proteomic analysis, we performed Co-IP coupled Western blot to detect those target proteins in the USP2a-immunoprecipitated complexes. As shown in **Figure 5D**, the seven target proteins were readily detected in the protein complexes of USP2a, thus solidifying the conclusion that those proteins are USP2a downstream target proteins.

Dysregulation of USP2a target proteins in the HCC subjects

As a deubiquitinating enzyme, one of the major USP2a activities is to regulate the target proteins through modulating protein stability. Since USP2a was upregulated in HCC subjects (**Figure 1**), we expected that the protein expression levels of the USP2a targets were also altered in HCC. Indeed, as shown in **Figure 6**, the protein levels of HSPA1A, DNAJA1, RUVBL1, TARDBP, PCNA and VDAC2 were significantly elevated in HCC-T when compared to the normal liver tissues. In addition, among those targets, the protein levels of RUVBL1, TARDBP, PCNA and


A
Normal
HCC-NT
HCC-T
70kDa
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GAPDH
Relative HSPA1A Protein Levels
*
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DNAJA1
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Relative DNAJA1 Protein Levels
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70kDa
TCP1
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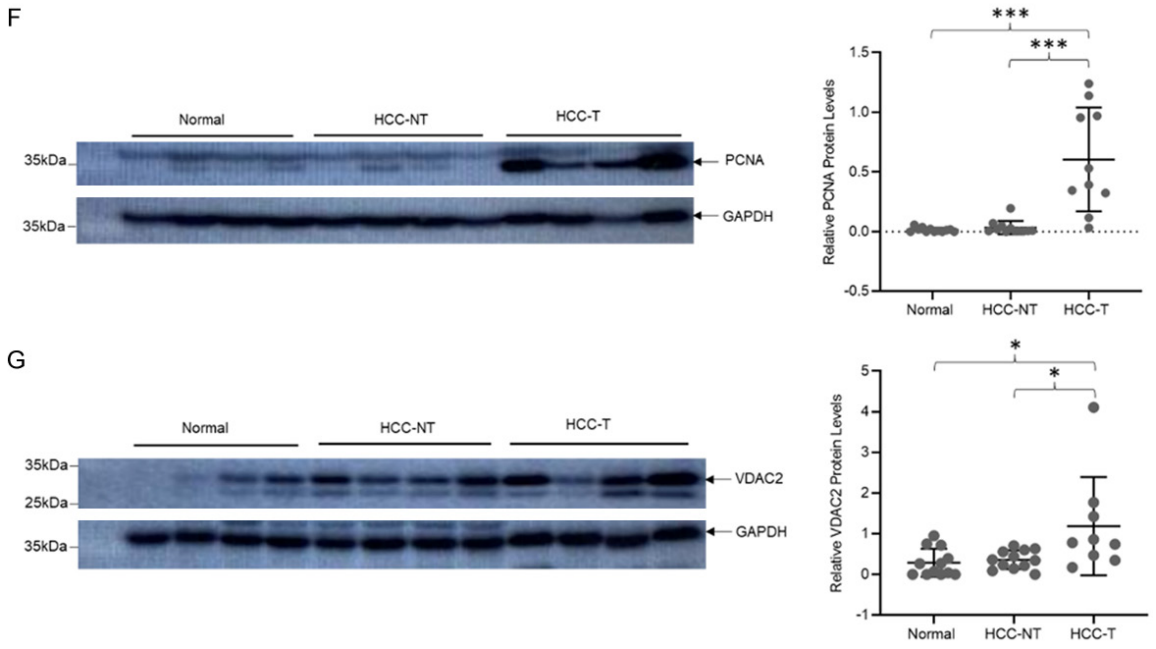

Figure 6. Dysregulated expression of USP2a target proteins in HCC subjects. (A) The expression levels of USP2a target proteins in human normal liver (n=12), HCC-NT (n=12) and HCC-T (n=9 or 10) tissues were detected and quantified by Western blot, including VDAC2, (B) HSPA1A, (C) DNAJA1, (D) RUVBL1, (E) TARDBP, (F) TCP1 and (G) PCNA. The results from four representative tissue samples from each of the three groups (normal, HCC-NT and HCC-T) were presented in the left panel. The Western blots were performed once or twice dependent on the quality of the results. $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$ in one-way ANOVA, followed by Tukey post-hoc test for multiple comparisons.

VDAC2 were also significantly upregulated in HCC-T when compared to the HCC-NT tissues. On the other hand, to our surprise, TCP1 protein levels were markedly down-regulated in HCC-T when compared to normal or HCC-NT tissues (**Figure 6F**). Taken together, the results demonstrated that those newly identified USP2a targets were dysregulated in the HCC subjects, thus potentially playing important roles in the pathogenesis of HCC.

Discussion

USP2a has been implicated in various diseases, particularly multiple types of cancers [32-34]. USP2a expression was markedly down-regulated in bladder cancer [32] while its expression was upregulated in gliomas [33] and prostate adenocarcinomas [34]. In this study, we found that USP2a expression was significantly upregulated at both mRNA and protein levels in HCC-T when compared to the normal liver tissues, which is consistent with previous studies [21, 26, 27]. 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 true normal status of the tissues. Indeed, our results revealed that USP2a expression exhibited an increasing trend from normal liver tissues to HCC-NT and HCC-T (**Figure 1A** and **1B**). 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. Compared to the HCC-NT tissues, USP2a expression was increased in HCC-T tissues but the differences did not reach statistical significance (**Figure 1A**, **1B**, **1D** and **1F**), mainly due to large individual variations or limited sample size. The USP2a expression was markedly upregulated in a large portion of the tumor samples but significantly reduced in some of other samples (**Figure 1C**, **1E** and **1G**). The data reflected the complexity of HCC pathogenesis and indicated that other factors than USP2a may play dominant roles in the development of those HCC tumors, such as individual genetic profile variations. Another remote possible explanation is that similar to USP2b, USP2a

may also have the ability to exert its oncogenic or tumor-suppressive activities depending on cellular contents.

HCC is often developed from chronic liver injuries such as cholestasis, hepatitis and cirrhosis. Elevated bile acids as a result of liver injuries are etiologically linked to the development and progression of HCC [24-27]. Young children carrying genetic defects in the bile salt export pump have elevated bile acids and develop HCC at early ages [25]. Consistently, elevated bile acid concentrations play an important role in the promotion and progression of HCC in rodents [26]. It is well established that high levels of bile acids are toxic to hepatocytes, causing cell death through either apoptosis or necrosis [28-31] with necrosis being the predominant mechanism for bile acid-induced cell death [30, 31]. Therefore, we chose CDCA as the apoptosis and necrosis inducer to investigate the effects of USP2a on bile acid-induced apoptosis and necrosis in hepatocytes. We found that knockout of USP2 made the USP2-KO cells significantly more susceptible to bile acid-induced apoptosis (**Figure 3A** and **3B**) and necrosis (**Figure 3C** and **3D**) than the HepG2 wt cells. On the other hand, forced expression of USP2a in USP2-KO cells markedly reduced bile acids-induced apoptosis and necrosis (**Figure 3E** and **3F**). The data thus, for the first time, firmly established that USP2a had potent anti-apoptotic and anti-necrotic activities in hepatocytes, which certainly contributes to the development of HCC. In line with our findings, silencing USP2a in prostate cancer cells induced apoptosis [34]. On the other hand, USP2a was reported to promote apoptosis in glioma cells [10] and Hela cells [35]. Therefore, it can be concluded that USP2a has the ability to exert its pro- or anti-apoptotic activities in a content or cell type-specific manner.

A previous study demonstrated that USP2a increased SK-Hep1 cell growth *in vivo* in a subcutaneously grafted, immunocompromised nude mouse model [21]. However, the cell line grafted models do not capture the features of naturally developed HCC tumors and tumor microenvironment as well as lack genomic heterogeneity associated with tumors originating *de novo* [36]. In this study, we investigated the effects of USP2a on *de novo* HCC development using a DEN-induced HCC mouse model. Consistent with the oncogenic activities of USP2a detected *in vitro*, including promoting cell proliferation and augmenting anti-apoptotic and anti-necrotic activities, overexpression of USP2a promoted *de novo* HCC development in mice with significantly increased tumor occurrence rates as well as tumor sizes (**Figure 4B** and **4D**). Consequently, the liver/body weight ratios were significantly increased in mice treated with USP2a (**Figure 4G**). Taken together, the consistent *in vitro* and *in vivo* data from this study firmly established that USP2a is an oncogene, playing important roles in the pathogenesis of HCC.

In this study, through unbiased Co-IP coupled proteomic analysis and western blot, we identified USP2a-specific target proteins that potentially mediate USP2a's oncogenic activities. Notably, seven target proteins are directly involved in cell proliferation, apoptosis, and pathogenesis of various cancers including HSPA1A, DNAJA1, TCP1, RUVBL1, TARDBP, PCNA and VDAC2. Among the seven targets, three proteins including HSPA1A, DNAJA1 and TCP1 are either chaperone (HSPA1A and TCP1) or co-chaperone (DNAJA1) and play important roles in protein folding, assembling, and trafficking. HSPA1A and DNAJA1 have been reported to promote cancer cell proliferation, metastasis, and resistance to apoptosis [37-39]. HSPA1A expression is directly associated with the prognosis of HCC [40] and breast cancer [41]. TCP1 has been associated with HCC malignancy, poor prognosis, and shorter overall survival of HCC patients [42-44]. Consistently, we found that the expressions of HSPA1A and DNAJA1 were significantly upregulated in HCC-T tissues (**Figure 6B** and **6C**). However, in contrast to previous reports [42, 44, 45], TCP1 expression was markedly down-regulated in HCC-T tissues (**Figure 6F**). Such up- and down-regulated expression phenomenon of TCP1 was also reported in breast cancer [46, 47]. The results indicated that dysregulation of TCP1 is associated with the pathogenesis of HCC. However, it remains to be determined the underlying mechanism by which USP2a down-regulates TCP1. Taken together, USP2a exerts its oncogenic activities, at least partially, by modulating protein folding, assembling, and trafficking through regulating its targets HSPA1A, DNAJA1 and TCP1.

The other three USP2a target proteins including RUVBL1, TARDBP and PCNA are involved in

DNA replication and transcription. RUVBL1 is a DNA-dependent ATPase and DNA helicase, playing important roles in DNA replication and gene transcription. TARDBP, also called transactive response DNA binding protein 43 kDa (TDP-43), participates in transcription and RNA splicing. PCNA is involved in DNA replication and damage repair. Dysregulation of those proteins has been implicated in the pathogenesis of various cancers [48-54]. Inhibition of RUVBL1 activity significantly reduces the proliferation of cancer cells [48-50]. Similarly, silencing of TARDBP expression significantly reduced HCC cell proliferation *in vitro* and *in vivo* [51]. Furthermore, TARDBP was reported to be a prognostic marker for HCC and its elevated expression was directly correlated with high grade and advanced stage of HCC [52]. Similarly, upregulated PCNA expression was associated with an unfavorable prognostic prediction for HCC patients [53]. Consistently, PCNA expression was directly correlated with poor 5-year survival rates and tumor grades or stages in cervical cancer, gliomas, and other cancers [54]. As expected and consistent with previous reports [48-54], the expression levels of the three target proteins were significantly increased in HCC tumors (**Figure 6**). Taken together, USP2a-mediated upregulation of RUVBL1, TARDBP and PCNA contribute to the pathogenesis of HCC through modulating DNA replication and transcription. Lastly, VDAC2 is a member of the voltage-dependent anion channel pore-forming family of proteins on the mitochondrial outer membrane and is involved in the mitochondrial apoptotic pathway. VDAC2 exhibits either pro- or anti-apoptotic activities depending on its interacting partner BAK or BAX and plays important roles in the pathogenesis of many cancers [55-57]. Consistent with being a USP2a target protein, VDAC2 expression was significantly upregulated in HCC tumors (**Figure 6G**). Therefore, modulating the mitochondrial apoptotic pathway through regulating VDAC2 is potentially one of the mechanisms by which USP2a exerts its anti-apoptotic activities.

In summary, USP2a was upregulated in HCC subjects and acted as an oncogene to promote cell proliferation and resistance to bile acids-induced apoptosis and necrosis as well as *de novo* HCC development *in vivo*. Further investigation identified novel USP2a target proteins mediating its oncogenic activity through modulating protein folding and assembling, DNA replication and transcription, and mitochondrial apoptotic pathways. The findings provided a molecular basis for developing interventions to treat HCC through targeting USP2a or its downstream pathways.

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Disclosure of conflict of interest

None.

Abbreviations

CDCA, chenodeoxycholic acid; Co-IP, coimmunoprecipitation; DEN, diethylnitrosamine; DMSO, dimethyl sulfoxide; DNAJA1, DNAJ heat shock protein family (Hsp40) member A1; FXR, Farnesoid X receptor; FXR-KO, FXR knockout; GAPDH, glyceraldehyde 3-phosphate dehydrogenase; HCC, hepatocellular carcinoma; HCC-NT, HCC adjacent non-tumor tissue; HCC-T, HCC tumor tissue; HSPA1A, heat shock protein family A (Hsp70) member 1A; KEGG, Kyoto encyclopedia of genes and genomes; MS/MS, tandem mass spectrometer; PCNA, proliferating cell nuclear antigen; RAB1A, Ras-related protein Rab-1A; RUVBL1, RuvB like AAA ATPase 1; TARDBP, TAR DNA binding protein; TCP1, T-Complex 1; TDP-43, transactive response DNA binding protein 43 kDa; USP2, Ubiquitin specific peptidase 2; USP2a, USP2 isoform a; USP2b, USP2 isoform b; USP2-KO, USP2 knockout; VDAC2, voltage-dependent anion channel 2; Wt, wild type.

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