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Hepatitis B virus X protein prevents apoptosis of hepatocellular carcinoma cells by upregulating SATB1 and HURP expression

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ABSTRACT

Protein X from hepatitis B virus (HBV) appears to play a critical role in the development of hepatocellular carcinoma (HCC). The hepatoma upregulated protein (HURP) is also upregulated in a majority of HCC cases, therefore suggesting that HURP represents an oncogene. In this study, we describe a link between the viral protein HBx, HURP, and the establishment of cisplatin chemoresistance in HCC cells. Hep3B cells which express HBx displayed increased levels of HURP mRNA and protein, and showed resistance to cisplatin-induced apoptosis. Knockdown of HURP in HBx-expressing cells reversed this effect and sensitized Hep3B cells to cisplatin. Interestingly, SATB1, a global gene regulator which is often overexpressed in malignant breast cancer, was also induced following expression of HBx. The anti-apoptotic effect of HBx was shown to require activation of the p38/MAPK pathway in Hep3B cells. In addition, the expression of survivin, an anti-apoptotic protein, was also upregulated by HBx in an HURP-dependent manner. Taken together, these results indicate that HBx activates the expression of HURP via the p38/MAPK pathway and the SATB1 protein, culminating with the accumulation of the anti-apoptotic protein survivin. Our findings illustrate the role of the viral protein HBx in preventing apoptosis during cancer progression and establishment of chemoresistance.

1. Introduction

Chronic hepatitis B virus (HBV) infections have been identified as a major risk factor for the development of hepatocellular carcinoma (HCC) [1–3]. Several lines of evidence suggest that the X protein (HBx) from HBV plays a role in the pathogenesis of HCC [3–6]. For instance, HBx is known to interact with numerous host proteins, including the tumor suppressor p53, the DNA-repair-protein DDB1, and various proteasome subunits [5]. Binding of HBx to p53 transactivates various target genes such as components of the transcription factor AP-1 [7–9]. Interaction of HBx with DDB1 suppresses DNA repair [10,11], thereby inducing either oncogenesis or apoptosis. Several studies have also demonstrated that DDB1 can stabilize HBx [12,13], therefore indicating that DDB1 may play a role in HBx-induced suppression of cell growth and death of HeLa cells following serum starvation [13]. HBx also interacts with the proteasome subunits PSMC1 and PSMC7, and suppresses proteasome activity [14,15]. In addition, the HBx protein exerts a variety of biological effects such as modulating cellular apoptosis in the presence of various additional stimuli [16–18]. The role of HBx during apoptosis, however, remains unclear, with some studies showing pro-apoptotic effects [19–23], while others observed mainly anti-apoptotic effects [17,24–27]. Importantly, an experimental animal model suggests that HBx confers resistance of hepatoma cells to the chemotherapeutic drug cisplatin [24]. Still, how HBx regulates cell survival responses to chemotherapy remains unclear.

Hepatoma upregulated protein (HURP) was first reported to be abundant in HCC based on an integrative bioinformatics approach to analyze sequence tags expressed by human liver [28]. HURP was considered as a stem cell marker, and was named Dlg7 in this context. In contrast, HURP was found to be undetectable in fully differentiated cells [29]. Earlier studies have shown that overexpression of HURP in differentiated cells increased cell growth and blocked apoptosis induced by serum starvation [28,30]. HURP also represents a potential cell cycle regulator, specifically acting at mitotic phase, and is implicated in the carcinogenesis of human cancer cells [28]. Gene expression analysis has revealed that HURP represented a prognosis marker able to distinguish between benign and malignant adrenocortical tumors [31,32]. Overall, these reports suggest that HURP possesses an anti-apoptotic

Abbreviations: CDDP, cisplatin; DAPI, 4′,6-diamidino-2-phenylindole; CMV, cytomegalovirus; GFP, green fluorescent protein; HBV, hepatitis B virus; HBx, hepatitis virus B X protein; HCC, hepatocellular carcinoma; HURP, hepatoma upregulated protein; Luc, luciferase; MAPK, mitogen-activated protein kinase; PARP, poly-ADP-ribose polymerase; PCR, polymerase chain reaction; RT, reverse transcription; SATB1, T-cell-specific transcription factor; shRNA, short-hairpin RNA.

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function, and that it is involved in tumor development. However, the mechanisms regulating HURP gene expression and its anti-apoptotic activity remain elusive.

SATB1, a nuclear matrix factor, is implicated in the organization of chromosome loop and gene regulation. At the genome level, SATB1 seems to play a role in the organization of transcriptionally poised chromatin [33]. This protein was first proposed to represent a mediator of apoptosis [34]. SATB1 phosphorylation also regulates the transcription of IL-2 in a T-cell activation model, and a similar mechanism may be implicated in the global gene regulation activity of SATB1 [35]. Recently, it has also been demonstrated that sumoylation-directed caspase-3 cleavage of SATB1 may act to either control gene expression during normal cellular function or to assist in rapid clearance of damaging immune cells [36]. Recent studies also suggest that SATB1 promotes metastasis, and that overexpression of SATB1 increases multidrug resistance in breast cancer cells [37,38]. However, the role of SATB1 in the regulation of cell survival is not well understood. In this study, we provide evidence that HBx upregulates both SATB1 and HURP, and we show that this pathway has an anti-apoptotic effect that increases chemoresistance of hepatic cancer cells to cisplatin.

2. Materials and methods

2.1. Cell lines and kinase inhibitors

Human HEK293, control and HBx-expressing Hep3B cell lines (H4/HBx and H13/HBx) [39] were maintained in Dulbecco's modified Eagle's medium (Gibco, Carlsbad, CA, USA) supplemented with 10% fetal bovine serum (Gibco), penicillin (100 U/ml; Gibco) and streptomycin (100 μg/ml; Gibco). Inhibitors of ERK (PD98059), p38 (SB203580), JNK (JNKII) and PKC (RO381220) kinases were purchased from Calbiochem (San Diego, CA, USA).

2.2. Plasmids, transfection, and infection of recombinant DNA

Hepatitis B virus X cDNA containing a full-length open reading frame was amplified by polymerase chain reaction (PCR) from the HBV genome as described [17]. A 1.3 kb Not I/Hind III HBx cDNA fragment was used for the construction of recombinant adeno-expression vector pAdEasy1-GFP-HBx and for cell infection as described [40]. The same strategy was applied to generate the Adeno-β-Gal vector used as control. pcDNA3-SATB1 was isolated by PCR amplification using primers designed based on the GenBank database sequences (SATB1 accession number: NM_001131010); it was inserted in the pGEM®-T Easy vector (Promega, Madison, WI, USA), before being sub-cloned in the pcDNA3 expression vector (Invitrogen, Carlsbad, CA, USA) using the restriction enzymes EcoRI and XhoI. The resulting pcDNA3-SATB1 plasmid was expressed under the control of the cytomegalovirus (CMV) promoter. The following primers were used to amplify SATB1: forward (linked to EcoRI restriction site), 5′-GAATTCATGGATCATTTGAACGAGGCA-3′; reverse (linked with XhoI), 5′-CTCGAGTCAGTCTTTCAAATCAGTATT-3′. Transfection of the pcDNA3-HURP (a gift from Dr. C.-K. Chou, Chang Gung University; see reference [28]) or pcDNA3-SATB1 plasmids was performed using Lipofectamine (Invitrogen) according to instructions from the supplier.

2.3. Gene knockdown by recombinant lentivirus-expressing shRNA

pLKO.1 plasmids expressing shRNA for the knockdown of HURP or SATB1 were purchased from the National RNAi Core Facility

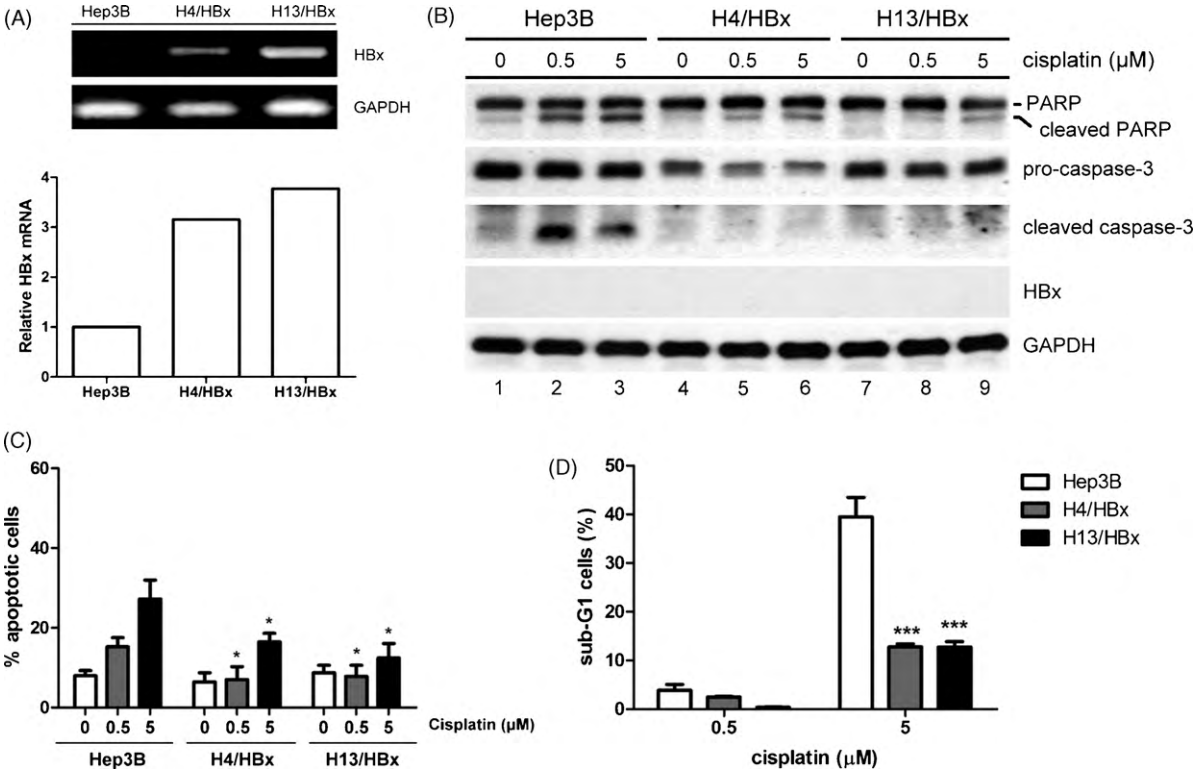

Fig. 1. Reduced cisplatin-induced apoptosis in Hep3B cells that express HBx. (A) Expression of HBx mRNA in H4/HBx and H13/HBx cells was monitored by conventional RT-PCR, and the PCR products were resolved on agarose gels (upper panel). GAPDH was used as a loading control here and throughout this study. Relative HBx mRNA levels were quantified by RT-PCR using the GeneTools analysis software of Syngene systems (lower panel). (B) Reduction of cisplatin-induced caspase-3 activation in HBx-expressing Hep3B cells. The cells were treated with cisplatin at the indicated concentrations for 72 h, and Western blot was performed against the proteins shown. (C) Reduction of cisplatin-induced apoptosis in HBx-expressing Hep3B cells. The content of apoptosis in cells treated as in (B) was assessed by nuclear phenotype analysis. (D) Reduction of cisplatin-induced sub-G1 cell accumulation in Hep3B cells expressing HBx. The results shown in (C) and (D) represent means ± standard deviation of experiments performed in triplicate. Statistical significance was expressed as $^{*}p < 0.05$, and $^{***}p < 0.001$ against control cells.

(Academia Sinica, Taipei, Taiwan). Knockdown efficiency of five plasmid clones for each gene was tested in HEK293 cells. Luciferase-shRNA (TRCN0000072244) was used as a negative control. Transient transfection was performed by adding 2 μg/well of shRNA plasmids along with 5 μl/well of Lipofectamine (Invitrogen) into cell suspensions kept in 6-well plates (1.5×10^4 cells/well). The primer sequences for the plasmids encoding shRNA used in this study were as follows: HURP, 5′-CCGGCATAAGGAATACGAACGAAATCTCGAGATTTCGTTCGTATTCCTTATGTTTTTG-3′, TRCN0000062232; SATB1, 5′-CCGGGCTCTATGTTATCCAAGCAATCTCGAGATTGCTTGGATAACATAGAGCTTTTT-3′, TRCN0000017173. Stable clones expressing the shRNA plasmids via lentivirus as vector were established in HCC cells. Recombinant lentivirus preparation and cell infection were performed according to the methods described by the supplier (National RNAi Core Facility).

2.4. RT-PCR

Quantitative RT-PCR was performed on total cellular RNA extracted as described previously [41]. GenBank sequence numbers were used to design primers for HBx (EU043345), HURP (NM_014750), SATB1 (NM_001131010), and GAPDH (NM_002046). PCR primer pairs were used at a concentration of 100 nM and were normalized against GAPDH. The following primers were used: HBx, forward, 5′-GCCCCGGGGCGATGGCTGCTCGGGTGTGCTG-3′; reverse, 5′-GCGTCGACTTAGGCAGAGGTGAAAAAGTTGCATG-3′; HURP, forward, 5′-CCCATCTTCCCTTGAGAAAG-3′; reverse, 5′-AGGAGACATCAAGAACATGC-3′; SATB1, forward, 5′-TGCAAAGGTTGCAGCAACCAAAAGC-3′; reverse, 5′-AACATGGATAATGTGGGGCGGCCT-3′; GAPDH, forward, 5′-TCCTGCACCACCAACTGCTT-3′; reverse, 5′-GAGGGGGCCATCCACGTCTT-3′. Each experiment included 30 reaction cycles (95 °C for 1 min; 55 °C for 30 s; 72 °C for 30 s), and the PCR products were separated by agarose gel electrophoresis. Relative mRNA levels were quantified using the GeneTools analysis software of Syngene systems (Cambridge, UK).

2.5. Western blot analysis

Fifty microgram of cell protein extract was separated on a 12% SDS-PAGE, transferred onto a PVDF membrane, and incubated with the primary antibodies directed against HBx (Affinity BioReagents, Dublin, OH, USA), SATB1, survivin, caspase-3, PARP, GFP, GAPDH (Santa Cruz Biotechnology, Santa Cruz, CA, USA), cleaved caspase-3 (Cell signaling Technology, Danvers, MA, USA), or HURP (a gift from Dr. C.-K. Chou).

2.6. Apoptosis and flow cytometry analysis

Apoptotic cells were determined based on their nuclear phenotype as described [39]. In addition to the activation of caspase-3 and PARP as monitored by Western blot, drug-induced caspase activation was evaluated by assessing the accumulation of sub-G1 cell population by flow cytometry [42].

2.7. Statistical analysis

Data were analyzed using a paired Student's *t* test to determine statistical significance and to compare the means of two different experimental groups. Data of experiments performed in triplicate were expressed as means ± standard deviation.

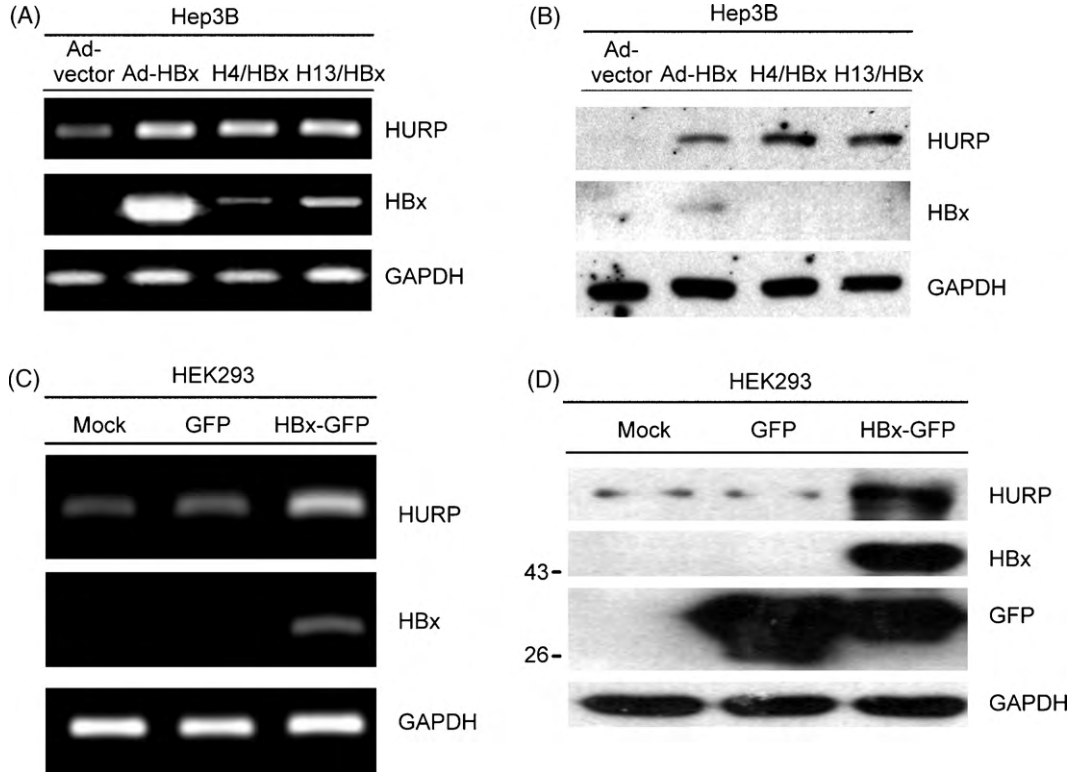

Fig. 2. Upregulation of HURP expression in Hep3B cells that transiently or stably express HBx. (A) HURP, HBx, and GAPDH mRNA levels were assessed by RT-PCR in Hep3B cells that transiently express the adenovirus vector (Ad-vector) or the vector coding for HBx (Ad-HBx). RT-PCR was also performed on Hep3B cells that stably express HBx (H4/HBx and H13/HBx). (B) Protein levels of the cells described in (A) and evaluated by Western blot showed increased HURP protein expression in HBx-expressing cells. (C) HEK293 cells were transfected with the vector coding for either GFP or GFP-HBx. mRNA levels of the indicated product was amplified by RT-PCR. (D) Protein levels of the cells described in (C) were assessed by Western blot analysis.

3. Results

3.1. Decreased cisplatin-induced apoptosis in hepatic cancer cells that express HBx

We first confirmed the expression of HBx in the Hep3B hepatic carcinoma cells H4/HBx and H13/HBx using RT-PCR (Fig. 1A). To assess whether HBx expression may increase chemoresistance to cisplatin, we treated the cells with a cytotoxic dose of cisplatin and monitored apoptosis by assessing cleavage of caspase-3 and its target poly (ADP-ribose) polymerase (PARP) (Fig. 1B). Reduced cleavage of both apoptosis markers was observed in HBx-expressing cells following cisplatin treatment when compared to control Hep3B cells (Fig. 1B). Although HBx mRNA was detected in H4/HBx and H13/HBx cell lines (Fig. 1A), the HBx protein was not detected in these conditions (Fig. 1B), probably due to its rapid degradation [43] by the proteasome [14,44]. Hep3B cells that stably express HBx also displayed significantly less apoptotic sub-G1 cells in response to cisplatin when assessed by flow cytometry (Fig. 1C and D). These results suggest that HBx may decrease the sensitivity of Hep3B cells to cisplatin by preventing apoptosis.

3.2. Upregulation of HURP expression in Hep3B cells that express HBx

HURP has been found to be overexpressed in HCC, suggesting that this protein represents an oncogene [28]. We observed that HURP mRNA transcript was induced by both the transient expression of HBx using an adenovirus vector (Ad-HBx) and the stable expression of HBx in Hep3B cells (Fig. 2A). Both kinds of HBx-expressing cells displayed around 2–3-fold HURP mRNA increase compared to control cells (Fig. 2A). While the HURP protein was not detected in control Hep3B cells, the protein was observed following both transient and stable expression of HBx (Fig. 2B). Overexpression of HBx using the adenovirus vector also

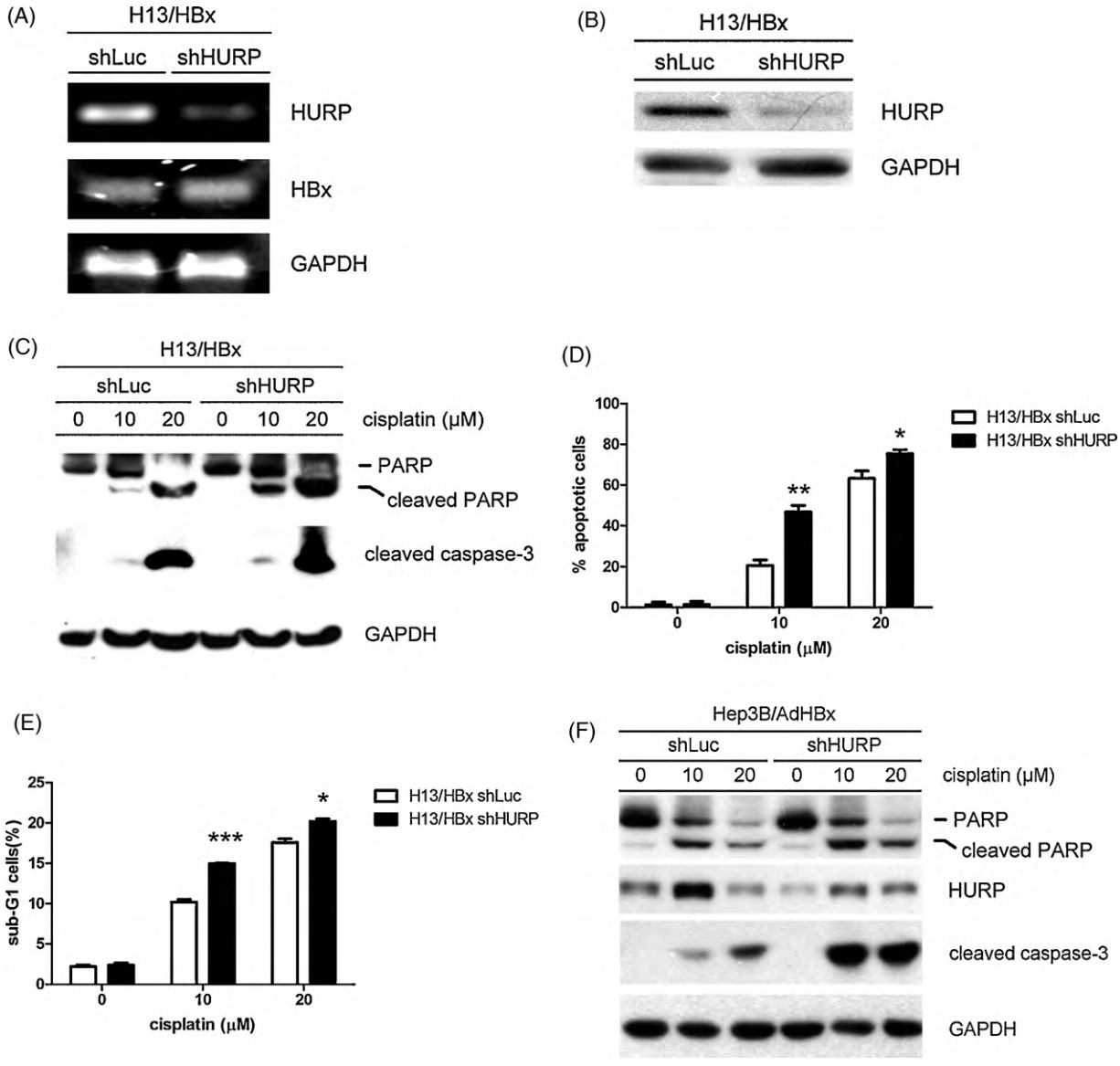

Fig. 3. HURP knockdown results in increased cisplatin-induced apoptosis in HBx-expressing Hep3B cells. (A) mRNA levels of HURP, HBx, and GAPDH was evaluated by RT-PCR following knockdown of HURP (shHURP) or luciferase (shLuc) in H13/HBx cells. (B) HURP knockdown efficiency was verified by Western blot. (C) Increased cisplatin-induced apoptosis in H13/HBx cells as determined by Western blot. The cells were treated with cisplatin at the indicated concentrations for 24 h. (D) Apoptosis levels in cells described in (C) were monitored by nuclear phenotype analysis. (E) Increased sub-G1 cell population following cisplatin treatment in shHURP-expressing cells. Cells described as in (C) were monitored by flow cytometry as described in the Section 2. (F) Hep3B cells that transiently expressed HBx were processed for Western blot analysis to estimate the levels apoptosis markers following treatments with either shLuc or shHURP as well as cisplatin at the indicated concentrations.

produced detectable amounts of HBx protein by Western blot (Fig. 2B). In this case, the weak correlation between HBx and HURP expression could also be due to the unstable state of HBx protein and to possible differences between transient (Ad-HBx) and stable (H4/HBx and H13/HBx) expression systems (Fig. 2A and B). To support the concept that HURP is induced by HBx, non-tumorigenic HEK293 cells were transfected with expression plasmids carrying either HBx-GFP fusion protein or GFP alone. We noticed a 2–3-fold increase in HURP mRNA in the presence of HBx compared to GFP control (Fig. 2C). Similarly, HBx, which was detectable in HEK293 following overexpression, also induced the accumulation of HURP at the protein level (Fig. 2D). These results indicate that the viral protein HBx is able to induce HURP expression in both HCC and HEK293 cells.

3.3. HURP knockdown is associated with increased cisplatin-induced apoptosis in Hep3B cells

To explore whether HURP expression also affects resistance to cisplatin, we used shRNA to knockdown HURP (shHURP) in

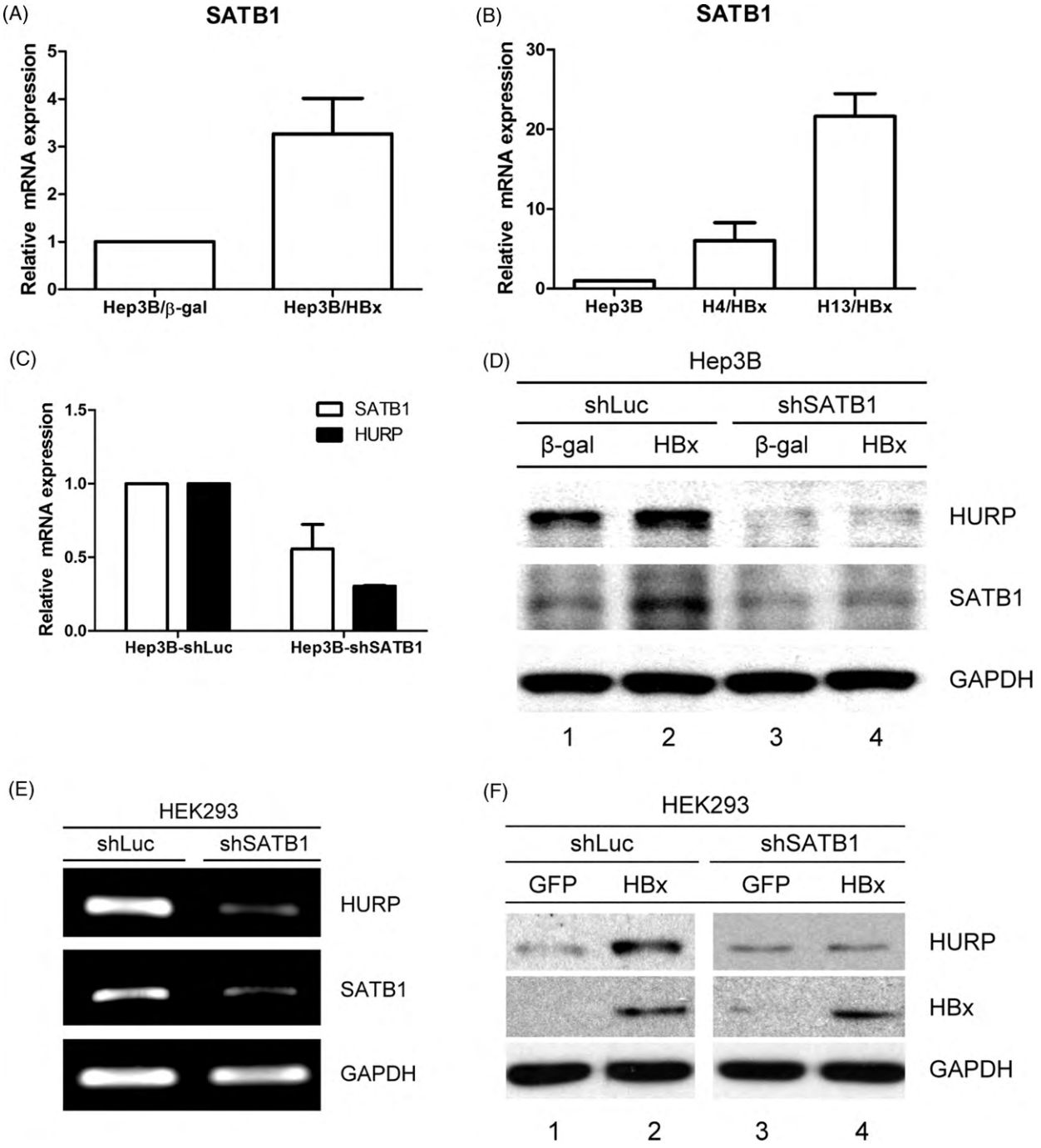

Fig. 4. Involvement of SATB1 in HBx-induced HURP accumulation. (A) Upregulation of SATB1 mRNA in Hep3B cells that transiently expressed HBx. The levels of SATB1 were monitored by quantitative RT-PCR. (B) Upregulation of SATB1 mRNA in Hep3B cells that stably expressed HBx. (C) Reduction of HURP mRNA following SATB1 knockdown in Hep3B cells. (D) Reduction of HBx-induced HURP protein following SATB1 knockdown in Hep3B cells. β-gal was used as a control against HBx expression. (E) Decreased HURP mRNA after SATB1 knockdown in HEK293 cells as assessed by RT-PCR. (F) Reduction of HBx-induced HURP protein following SATB1 knockdown in HEK293 cells.

HBx-expressing Hep3B cells (H13/HBx). Luciferase-shRNA (shLuc) was used as control. Following treatments with shRNA, HURP was partially decreased at both the mRNA (Fig. 3A) and the protein level (Fig. 3B). Following exposure to high cytotoxic concentrations of cisplatin (10 and 20 μM), cleavage of caspase-3 and PARP was increased in shHURP-expressing cells compared to controls (Fig. 3C). Following exposure to low cytotoxic concentrations of cisplatin (0.5 and 1 μM) as used above, we only detected increase of PARP cleavage in shHURP-expressing cells compared to controls (data not shown). Consistent with these results, apoptotic cells were increased following cisplatin treatment in shHURP cells (Fig. 3D). HURP knockdown also caused the accumulation of cisplatin-induced sub-G1 cells (Fig. 3E). Again, sensitization to cisplatin was more pronounced in cells exposed to 10 μM of cisplatin compared to those exposed to 20 μM (Fig. 3D and E). In addition, increased cisplatin-induced apoptosis was observed in Hep3B cells that transiently overexpressed HBx (Fig. 3F). These results indicate that HURP knockdown sensitizes HCC cells to cisplatin.

3.4. Involvement of SATB1 in HBx-induced HURP accumulation

A recent study showed SATB1 functions as a transcription factor and is upregulated in cases of malignant breast cancer with poor prognosis [37]. Based on the cDNA microarray analysis data published earlier (dataset on the GEOwebsite; accession number GSE5417; see also reference [37]), we found that HURP was upregulated by SATB1. This observation raised the possibility that HURP induction by HBx may be dependent on SATB1. Interestingly, we found that SATB1 mRNA accumulated at high levels in Hep3B cells that either transiently (Fig. 4A) or stably (Fig. 4B) expressed HBx. We monitored the expression of HURP in Hep3B cells following knockdown of SATB1 and found that this treatment reduced HURP at both the mRNA (Fig. 4C) and the protein level (Fig. 4D). We noticed that SATB1 and HURP protein levels slightly increased following ectopic expression of HBx in Hep3B cells compared to the β-galactosidase (β-gal) control (Fig. 4D, compare lanes 2 and 1). Following knockdown of SATB1, the HURP protein

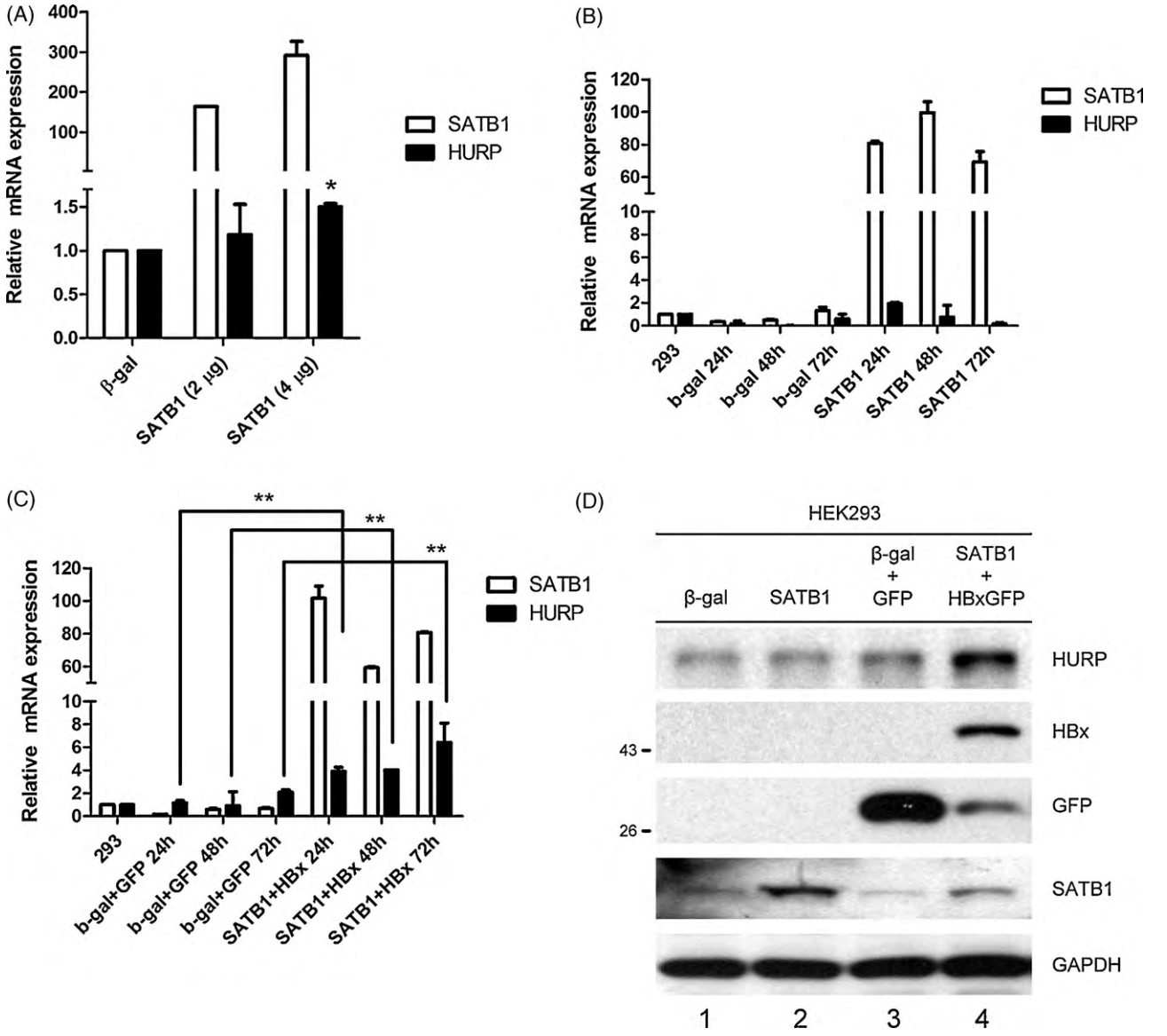

Fig. 5. HURP expression is increased by co-expression of SATB1 and HBx in HEK293 cells. (A) Expression of HURP mRNA following transfection of SATB1 plasmids in HEK293 cells was monitored by quantitative RT-PCR. The cells were transfected with 2 or 4 μg of either SATB1 or β-gal plasmid, followed by incubation for 18 h. (B) Negligible induction of HURP mRNA after SATB1 overexpression. The cells were transfected with 2 μg of either SATB1 or β-gal plasmid and were then incubated for the time indicated. (C) HURP expression was increased followed co-expression of SATB1 and HBx in HEK293 cells. The cells were treated with the indicated plasmids for extended periods of time prior to RT-PCR analysis. (D) Induction of HURP protein following co-expression of SATB1 and HBx as assessed by Western blot.

profoundly decreased in HBx-expressing Hep3B cells (Fig. 4D, compare lanes 4 and 2). Furthermore, SATB1 knockdown partially suppressed HURP mRNA in HEK293 cells when compared to shLuc control (Fig. 4E). While induction of HURP by HBx was intense in HEK293 cells (Fig. 4F, compare lanes 1 and 2), the level of HBx-induced HURP protein was considerably reduced following knockdown of SATB1 (Fig. 4F, compare lanes 4 and 2). These results suggest that SATB1 may be required for the induction of HURP by HBx.

3.5. Enhancement of HBx-induced HURP expression by SATB1 in HEK293 cells

Since SATB1 appears to be required for the induction of HURP by HBx, overexpression of SATB1 may result in the enhancement of HBx-induced HURP expression. To test this hypothesis, we transfected different amounts of SATB1 cDNA in HEK293 cells. While the SATB1 mRNA level was overexpressed in a dose-dependent manner up to 300-fold following plasmid transfection, HURP mRNA was only slightly increased up to 1.5-fold in these cells (Fig. 5A). Since HURP is presumed to act downstream of SATB1, SATB1-transfected cells were incubated for extended periods of time. While a considerable 100-fold increase of SATB1 mRNA was detected in transfected cells after 48 h, this treatment produced a less than two-fold increase of HURP mRNA (Fig. 5B). Interestingly, HURP mRNA was considerably upregulated when HBx-GFP was co-transfected along with SATB1 in HEK293 cells, compared to co-transfection of β-gal and GFP controls (Fig. 5C). Consistently, HURP protein level increased over two-fold following co-transfection of SATB1 and HBx-GFP plasmids (Fig. 5D, compare lanes 4 and 3). These observations support the concept that SATB1, with the help of HBx, can upregulate HURP expression at both the mRNA and the protein levels.

3.6. Involvement of p38/MAPK in HBx- and SATB1-induced HURP accumulation in Hep3B cells

To further assess the mechanism responsible for the upregulation of HURP by HBx and SATB1, we treated Hep3B cells which transiently express HBx with inhibitors of mitogen-activated protein kinase (MAPK) or protein kinase C (PKC) (see Section 2). Transient expression of HBx considerably induced HURP accumulation compared to β-gal control (Fig. 6A, compare lanes 2 and 1). Among the kinase inhibition treatments performed, p38 inhibitor (p38i) was found to profoundly suppress HURP induction by HBx (Fig. 6A, compare lanes 4 and 2). A similar experiment without removal of PKCi following pretreatment showed dramatic accumulation of HURP in these cells (Fig. 6A, lanes 7–9). Accordingly, PKC has been shown to negatively regulate SATB1 transactivation activity [35]. These results suggest that SATB1-dependent HURP protein expression was enhanced by PKCi treatments. In addition, the induction of SATB1 mRNA by HBx (over 3-fold) was almost totally suppressed to basal level following treatment with p38i (Fig. 6B). To gain further insights about the effects of p38 inhibition on cisplatin sensitivity, we treated Hep3B cells that stably express HBx (H4/HBx) with cytotoxic doses of cisplatin, and we monitored signs of apoptosis (Fig. 6C). We observed an enhanced cisplatin-induced cleavage of caspase-3 and PARP by treatment with p38i in H4/HBx cells (Fig. 6C). Consistent with the reduction in mRNA level

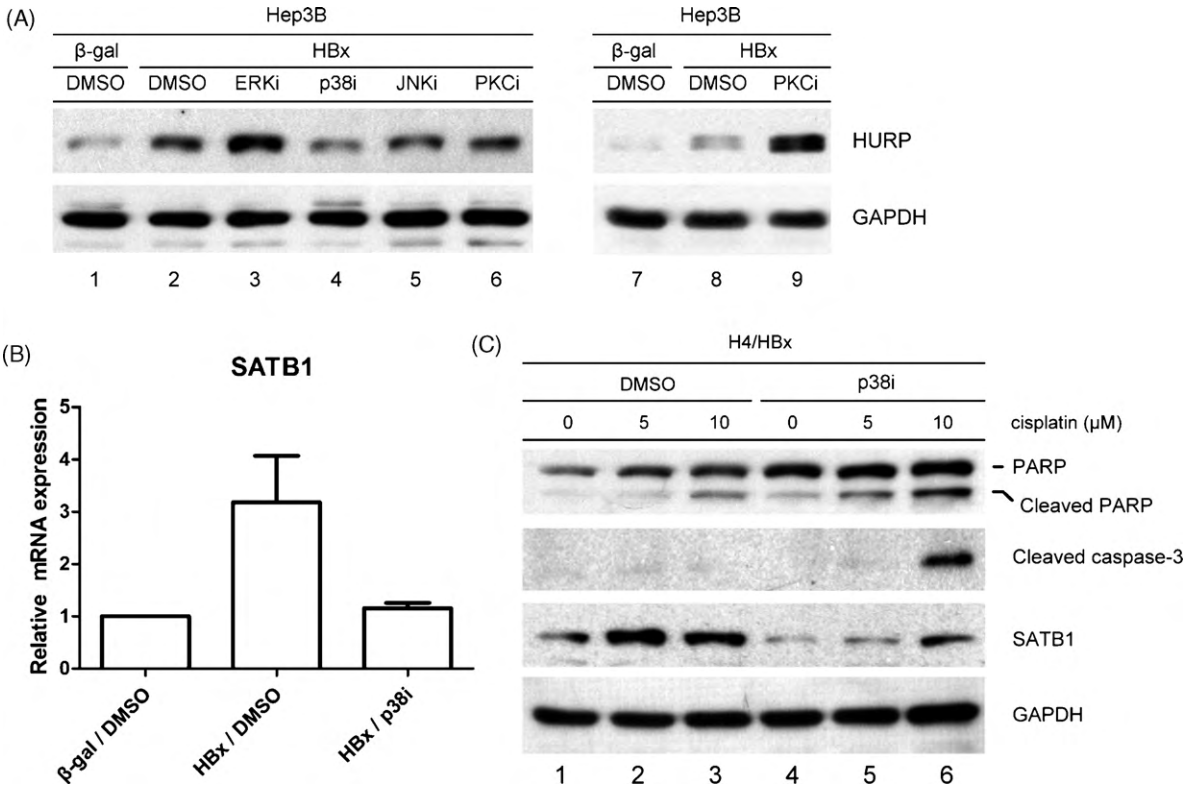

Fig. 6. p38/MAPK mediates HURP accumulation in HBx-expressing Hep3B cells. (A) Inhibition of HURP protein by p38/MAPK inhibitor in cells which transiently overexpressed HBx. 1×10^6 Hep3B cells were pre-treated with the indicated kinase inhibitors at a concentration of 10 μM for 2 h. In addition, a similar experiment performed without removal of the PKC inhibitor showed dramatic accumulation of HURP in these cells (lanes 7–9). Following infection with adenovirus-β-gal or adenovirus-HBx, the cells were then maintained for 48 h in the presence of 0.25 μM of the same inhibitors prior to Western blot analysis. (B) Decreased SATB1 mRNA following p38/MAPK inhibitor treatment in HBx-overexpressing Hep3B cells. Relative mRNA levels were monitored by quantitative RT-PCR. (C) Enhanced cisplatin-induced caspase-3 and PARP activation following treatments with the p38/MAPK inhibitor in H4/HBx cells. The cells were treated with cisplatin at the concentrations indicated for 48 h prior to Western blot analysis. ERKi, ERK inhibitor; p38i, p38 inhibitor; JNKi, JNK inhibitor; PKCi, PKC inhibitor.

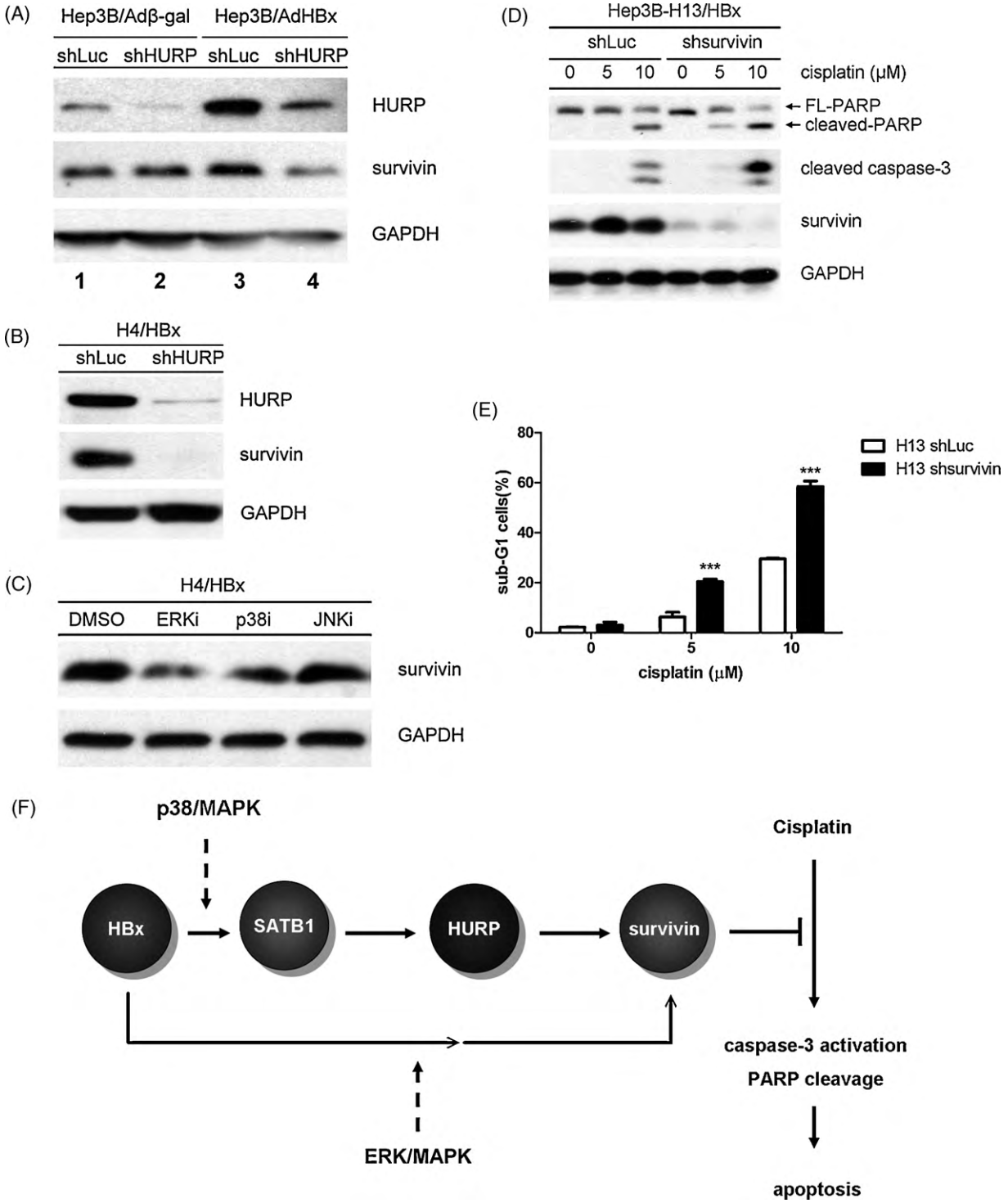

Fig. 7. Induction of survivin following expression of HBx and HURP in Hep3B cells. (A) Reduction of survivin protein levels following HURP knockdown in Hep3B cells that transiently expressed HBx. The cells infected with the indicated adenovirus constructs were treated with shLuc or shHURP, prior to Western blot analysis. (B) H4/HBx cells that expressed HBx showed reduction of survivin after knockdown of HURP. (C) Reduction of survivin following p38/MAPK inhibition. 1×10^6 H4/HBx cells were treated with the indicated kinase inhibitors at a concentration of 10 μM for 24 h and processed for Western blot. (D) Enhancement of cisplatin-induced caspase-3 activation and PARP cleavage in H13/HBx cells following treatment with survivin shRNA. (E) Reduction of cisplatin-induced sub-G1 cell accumulation in H13/HBx cells following survivin knockdown. The results represent means ± standard deviation of experiments performed in triplicate. Statistical significance was expressed as $^{***}p < 0.001$ against control cells. (F) Proposed model to explain the link between the viral protein HBx, the p38/MAPK pathway, SATB1, HURP, and survivin in mediating anti-apoptotic effects during cisplatin treatment. Another less defined ERK/MAPK pathway which may regulate survivin independently of HURP is also shown.

(Fig. 6B), the protein level of SATB1 was also suppressed following treatment with p38i (Fig. 6C). These results suggest that p38 may be involved in the induction of SATB1 and HURP by HBx, and that the p38 pathway may mediate chemoresistance to cisplatin in HCC cells.

3.7. Induction of survivin following HBx-induced HURP expression in Hep3B cells

Although HURP is upregulated by HBx and is implicated in protecting cells against apoptosis, the factors mediating this

effect are currently unknown. Survivin is an anti-apoptotic protein known to be induced by MAPK signals in cells overexpressing HBx [45]. To explore this issue, we examined Hep3B cells that transiently or stably express HBx to determine whether survivin expression is regulated by HURP knockdown. We found that survivin protein level was indeed induced by transient expression of HBx (Fig. 7A, compare lanes 3 and 1). HURP knockdown in HBx-expressing cells considerably reduced the level of survivin (Fig. 7A, compare lanes 4 and 3). Similarly, HURP knockdown also dramatically suppressed the protein level of survivin in H4/HBx cells that stably express HBx when compared to control (Fig. 7B). Furthermore, survivin protein level was also reduced by inhibiting p38 and ERK in H4/HBx cells (Fig. 7C). In order to assess whether survivin is the major downstream target for the protective role of HBx, we suppressed survivin expression using shRNA, and monitored signs of apoptosis. Indeed, cisplatin-induced cleavage of caspase-3 and PARP was enhanced by survivin knockdown in H13/HBx cells (Fig. 7D, compare lanes 4–6 and lanes 1–3). H13/HBx cells expressing survivin shRNA also displayed significantly more apoptotic sub-G1 cells in response to cisplatin when assessed by flow cytometry (Fig. 7E). These results suggest that HBx decreases the sensitivity of Hep3B cells to cisplatin by preventing apoptosis. Overall, our results suggest a novel working model for the anti-apoptotic role of HBx during HCC progression and chemoresistance to cisplatin (Fig. 7F). In this model, the HBx protein produced during HBV hepatocyte infection elicits SATB1 expression in a p38-dependent manner, thereby resulting in the induction of HURP. Afterwards, HURP induces the anti-apoptotic protein survivin which in turn blocks the apoptotic response induced by cisplatin.

4. Discussion

In this study, we found that the viral protein HBx from HBV has an anti-apoptotic effect on HCC cells, eventually leading to cisplatin chemoresistance. The increased chemoresistance of HBx-expressing HCC cells was associated with the activity of STAB1, HURP, and survivin proteins (Fig. 7E). Earlier studies have shown that the transactivation activity of SATB1 is regulated negatively by PKC [35]. We observed that the anti-apoptotic action of HBx requires SATB1 and the p38/MAPK pathway. As such, HBx may play a role by either upregulating p38/MAPK and SATB1 activity or by directly upregulating HURP transcription. High levels of HURP favored the expression of the anti-apoptotic survivin in HBx-expressed cells. Although inhibition of ERK also reduced the induction of survivin (this study; see also reference [45]), HURP levels were not affected by ERKi (Fig. 6A), suggesting that HBx may induce survivin through another pathway requiring ERK. Our finding may partly explain the anti-apoptotic role of HBx in the development of HBV-associated HCC. In line with this concept, it has been demonstrated that stable expression of HBx is also able to suppress TGF-β-induced apoptosis in Hep3B cells by stimulating PI3-kinase activity [17,26].

Additionally, earlier studies showed that HBx can overcome the anti-apoptotic effect of Bcl-2 against Fas cytotoxicity in mouse liver [46]. HBx also sensitized UV-irradiated liver cells by activating fas ligand gene [19]. In some studies, HBx enhanced Wnt/β-catenin pathway, and was considered to be one of the main driving forces of hepatocarcinogenesis, as seen for instance, in Huh7 and Hep3B cells which express point and deletion mutant p53 but promotes the degradation of β-catenin via activation of Siah-1 ubiquitin ligase in wild-type p53 HepG2 [47,48]. One possible explanation for the divergent results obtained from the studies on the pro/anti-apoptotic effects of HBx may be dependent on the status of p53.

Earlier, we identified HURP and SATB1 as representing HBx-upregulated genes in HCC cells. Interestingly, SATB1 is rapidly induced by HBx, while HURP takes a longer time before it can be upregulated to appreciable levels. This observation could be explained by the finding that phosphorylation of SATB1 by p38/MAPK at the appropriate locations and levels may be required for transactivation of the HURP gene in HCC cells. As such, HBx-directed modification on SATB1 could exert transactivation of HURP which would then become a novel target with a potential biological significance in HCC. In addition, HBx has been reported to play a role in centrosome dynamics and mitotic spindle formation by interacting with different cellular partners implicated in these processes [49]. The reported function of HURP also appears to be closely related to microtubule stabilization and spindle organization [28]. Our data may provide a link between HBx expression, HURP, and chromosomal instability in HBV-related carcinogenesis. As such, the simplified model presented here implies a sequential involvement of various factors during the progression of HCC and the emergence of chemoresistance. Our report provides a promising molecular pathway to explain the action of HBx in preventing apoptosis of hepatic cancer cells during chemotherapy.

Conflicts of interest

The authors declare no conflict of interest.

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