Wip1 contributes to the adaptation of HepG2 human liver cancer cells to stress hormone-induced DNA damage

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Received March 31, 2022; Accepted October 19, 2022

DOI: 10.3892/ol.2022.13617

Abstract. Numerous studies have shown that the release of stress hormones resulting from repeated exposure to chronic psychological stress increases DNA damage and promotes tumorigenesis. However, the mechanisms that enable cancerous cells adapt to stress hormone-induced DNA damage and survive remain unclear. The present study aimed to investigate the impact of stress hormones on the survival of liver cancer cells and the underlying mechanism. HepG2 human liver cancer cells were treated with dexamethasone (DEX), epinephrine (EPI) and norepinephrine (NE) and subjected to the testing of DNA damage, cell survival and cell apoptosis by alkaline comet assay, CCK-8 viability assay and flow cytometry, respectively. The protein expression levels of DNA damage response factors were determined by western blotting analysis. The results revealed that treatment of HepG2 cells with DEX, EPI and NE induced DNA damage without affecting cell survival or inducing apoptosis. The protein levels of wild-type p53-induced phosphatase 1 (Wip1), a type 2C family serine/threonine phosphatase, were increased, and the dephosphorylation of DNA damage response factors, including phosphorylated (p-)ataxia-telangiectasia mutated and p-checkpoint kinase 2, occurred following treatment with DEX, EPI and NE. In addition, a cycloheximide chase assay was performed to explore the protein stability under treatment with stress hormones. Compared with vehicle-treated cells, Wip1 exhibited increased protein stability in stress hormone-treated HepG2 cells. Eventually, the depletion of Wip1 using small interfering RNA verified the role of Wip1 in the modulation of stress hormone-induced DNA damage. These findings suggest that cancerous cells likely adapt to stress hormone-induced DNA damage via Wip1 upregulation. The present study provides an insight into the underlying mechanism that links chronic psychological stress with tumor growth and progression.

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Abbreviations: ATM, ataxia telangiectasia mutated; ATR, ataxia telangiectasia and Rad3 related; Chk1, checkpoint kinase 1; Chk2, checkpoint kinase 1; DEX, dexamethasone; DMSO, dimethyl sulfoxide; EPI, epinephrine; FBS, fetal bovine serum; γ-H2AX, phosphorylated histone 2AX; HPA axis, hypothalamic-pituitary-adrenocortical axis; NE, norepinephrine; PCR, polymerase chain reaction; PI, propidium iodide; SNS, sympathetic nervous system; Wip1, wild-type p53-induced phosphatase 1

Key words: Wip1, PPM1D, stress hormone, adaptive DNA damage, liver cancer

Introduction

Numerous studies have reported an association between psychological stress and the overall survival of patients with various cancers, including breast, liver, lung, pancreatic, prostate, colon and ovarian cancers (1-7). Some studies have shown important cellular mechanisms that link the stress responses of the hypothalamic-pituitary-adrenocortical (HPA) axis or the sympathetic nervous system (SNS) with cancer progression and metastasis (8-10). Under conditions of psychological stress, the HPA axis is activated, which leads to the release of corticotrophin-releasing hormone from the hypothalamus and the secretion of adrenocorticotrophic hormone from the anterior pituitary. As a result, stress-associated hormones such as cortisol and catecholamines including epinephrine (EPI) and norepinephrine (NE) are released from the adrenal gland. These hormones exert strictly controlled effects, including the elevation of blood pressure, heart rate and blood sugar level, that prime an individual to respond to a perceived threat. In addition to the HPA axis, the central SNS is directly associated with the regulation of the stress response via the release of catecholamines from autonomic nerve endings (11). In contrast to the transient and strictly controlled stress response, exposure to sustained stress adversely affects tumor progression

and cancer therapy; dysregulation of the HPA axis has been shown to have a detrimental effect on cancer incidence and survival. For example, in one study, patients with hepatocellular carcinoma (HCC) had significantly higher serum cortisol levels than healthy subjects (12). In another study, patients with breast cancer were reported to have high serum cortisol levels, which could be suppressed by emotional support (13). Glucocorticoids can reach the tumor microenvironment via the blood circulation and act on the glucocorticoid receptors expressed by several types of cancer cells to regulate diverse cellular signaling pathways. They thereby increase the ability of cancer cells to proliferate and invade and affect the interaction between cancer cells and their microenvironment to promote tumor progression and metastasis (14-16). Similarly, catecholamines can contribute to stress-induced cancer growth and metastasis via adrenergic receptors located on the surface of cancer cells, leading to the activation of Ras/extracellular signal-regulated kinase, NF-κB and cAMP-dependent protein kinase pathways, which regulate cellular responses including proliferation, differentiation and apoptosis (17,18). In a study of women with triple-negative breast cancer treated with neoadjuvant chemotherapy, the patients treated with β-blockers that interfere with the receptor binding of EPI and other stress hormones exhibited improved relapse-free survival compared with women who did not receive β-blocker treatment (19). It is evident that surges in stress hormone levels facilitate the development and progression of various cancers.

Although there is considerable evidence associating psychosocial stress with cancer development and progression, the mechanism is not fully understood. One possible mechanism is that exposure to stress and stress hormones such as cortisol and catecholamines increases DNA damage and promotes tumorigenesis (20,21). A study demonstrated that the incubation of 3T3 mouse fibroblasts with EPI or NE resulted in long-term DNA damage that was sufficient to induce genomic instability and vulnerability to tumor transformation (22). Moreover, another study revealed that physiological concentrations of cortisol induced DNA damage in cells, which was associated with the transcriptional upregulation of DNA damage sensors, including checkpoint kinase 1 (Chk1) and Chk2 and the cell cycle regulator gene cell division cycle 25A (20). Consequently, long-term effects on genomic stability result in increased cell transformation and/or tumor formation. Additionally, numerous studies have demonstrated that stress increases the growth of existing tumors and the risk of tumor metastasis (23-25). Proposed mechanisms for these stress effects include increases in VEGF and angiogenesis resulting from the activation of β-adrenergic pathways (26). Stress hormones have also been shown to promote the migration and invasion of malignant cells via the elevation of matrix metalloproteinases, which are known for their ability to degrade the extracellular matrix and facilitate cell invasion (27,28). Hence, stress contributes to the initiation, growth and metastasis of tumors.

However, it appears contradictory that the stress response induces DNA damage but also promotes the growth and metastasis of existing tumors. We hypothesize that cancer cells undergo DNA damage adaptation to survive stimulation by chronic stress; DNA damage adaptation is a process by which cancer cells adapt to DNA damage and allow cell division despite the presence of unrepaired DNA damage (29,30). Various oncogenic signaling pathways are also activated to promote uncontrolled tumor growth in this process. The adaptation to DNA damage is mediated by checkpoint recovery process effectors, which are considered oncogenes. Wild-type p53-induced phosphatase 1 (Wip1), also known as protein phosphatase, Mg^{2+}/Mn^{2+} dependent 1D or protein phosphatase 2Cδ, is a type 2C family serine/threonine phosphatase that contributes to the inactivation of checkpoint recovery by removing DNA damage-induced phosphorylation in several of its components, including p53, ataxia-telangiectasia mutated (ATM), ataxia telangiectasia and Rad3 related (ATR), Chk1, Chk2 and phosphorylated histone 2AX (γ-H2AX) (31). Wip1 was first identified as a p53 target gene, which negatively regulates the function and stability of p53 following cellular stress (32). In a study of Fanconi anemia, a chromosomal instability syndrome characterized by bone marrow failure and a predisposition to cancer, Fanconi anemia cells with a large amount of DNA damage continued to undergo cell division after the induction of DNA damage by ignoring the presence of unrepaired DNA damage, while the inhibition of Wip1 prevented cell cycle progression and division (33). Therefore, we hypothesize that the upregulation of Wip1 preserves the capacity of cells to divide when stress hormones induce DNA damage in cancer cells.

Liver cancer is among the most common cancers in China and >50% of the liver cancer cases and deaths worldwide are estimated to occur in China (34). However, the effects of stress response on liver cancer are yet to be determined. A previous study demonstrated that the upregulation of Wip1 expression is associated with progressive pathological features and a poor prognosis in patients with HCC (35). In addition, Xu *et al* reported that Wip1 was highly expressed in ~59% of patients with HCC and its upregulation was an independent predictor of HCC-specific overall survival (36). With the aim of investigating the impact of stress hormones on the survival of liver cancer cells, the present study examined the expression of Wip1 in HepG2 cells treated by stress hormones and identified the role of Wip1 in the DNA damage and apoptosis induced by stress hormone stimulation.

Materials and methods

Chemicals and reagents. Dexamethasone (DEX), EPI and NE were purchased from Target Molecule Corp. All compounds were dissolved in dimethyl sulfoxide (DMSO) to a concentration of 10 or 20 mM and preserved at -20˚C. DMSO served as the vehicle control in all experiments. The antibody against Wip1 was obtained from Abcam (cat. no. ab31270; dilution 1:1,000). Antibodies against ATM (cat. no. 2873), phosphorylated (p-)ATM (Ser1981) (cat. no. 5883), Chk2 (cat. no. 2662), p-Chk2 (Thr68) (cat. no. 2197), p-p53 (Ser15) (cat. no. 9287), p53 (cat. no. 9282), γ-H2AX (cat. no. 9718) and H2AX (cat. no. 7631), and a second primary antibody against Wip1 (cat. no. 11901) were purchased from Cell Signaling Technology, Inc. Antibodies against β-actin (cat. no. AC026) and GAPDH (cat. no. AC033) were obtained from ABclonal Biotech Co., Ltd. All antibodies were used at the dilutions recommended by the manufacturer unless otherwise specified.

Cell culture and cell viability assay. The HepG2 human liver cancer cell line (a gift from Dr Jingrong Cui at Peking University, Beijing, China) was cultured in Eagle's minimum essential medium (Macgene™; M&C Gene Technology) supplemented with 10% (v/v) fetal bovine serum (FBS; PAN-Biotech GmbH), streptomycin (100 μg/ml; Macgene; M&C Gene Technology) and penicillin (100 U/ml; Macgene; M&C Gene Technology) in a 5% CO_2 incubator at 37˚C. To determine the cytotoxicity of DEX, EPI and NE, HepG2 cells were treated with or without each of these compounds for 72 h in a 5% CO_2 incubator at 37˚C followed by a cell viability assay using Cell Counting Kit-8 (Dojindo Laboratories, Inc.) according to the manufacturer's instructions.

Transient transfection. The small interfering RNAs (siRNAs), namely Wip1 siRNA (cat. no. sc-39205) and control siRNA (cat. no. sc-36869 or sc-37007), were purchased from Santa Cruz Biotechnology, Inc. Briefly, 40 μl of 10 μM siRNA duplex was diluted with 400 μl siRNA Transfection Medium (Santa Cruz Biotechnology, Inc.) and then mixed with 30 μl siRNA Transfection Reagent in 400 μl siRNA Transfection Medium followed by incubation for 40 min at room temperature. In a 10-cm tissue culture dish of ~80% confluent cells, each transfection was conducted by washing with 2 ml of siRNA Transfection Medium and adding 3.2 ml siRNA Transfection Medium and 800 μl transfection mixture. After incubation for 5 h at 37˚C in a 5% CO_2 incubator, cells were supplemented with 4 ml normal growth medium containing two times the normal serum and antibiotics concentration without removing the transfection mixture, and incubated for an additional 18 h followed by cell reseeding for the subsequent experiments.

Alkaline comet assay. The alkaline comet assay was performed using the Trevigen CometAssay® Kit (Trevigen, Inc.; Bio-Techne) according to the manufacturer's instructions. Briefly, HepG2 cells were seeded at $8x10^4$ cells/well in a 12-well plate. After 24 h, the cells were treated with vehicle control, 5 μM DEX, 1 μM EPI or 1 μM NE for 4 h in a 5% CO_2 incubator at 37˚C. Then the cells were harvested, washed twice with phosphate-buffered saline (PBS) and mixed with 1% low-melting agarose in PBS at 37˚C to form a single cell suspension embedded in agarose. The resulting mixture was immediately pipetted on with the sample area of a CometSlide (Trevigen, Inc.; Bio-Techne). The slides were placed at 4˚C for 10 min and then lysed at 4˚C overnight in the dark. After lysis, the slides were subjected to electrophoresis at 21 V for 45 min at 4˚C, and then immersed twice in distilled water for 5 min and once in 70% (v/v) ethanol for 5 min. The slides were dried completely at 37˚C for 10-15 min and then stained with propidium iodide (PI) for 10 min at room temperature. Comets were observed using an Olympus BX53 fluorescence microscope (Olympus Corp.) equipped with an Olympus DP72 camera (Olympus Corp.). The percentage of DNA in the tail was quantified based on ≥30 randomly selected cells in each sample using Image J software (version 1.53c; National Institutes of Health) as previously described (37).

Cell apoptosis assay. HepG2 cells were seeded at $2x10^5$ cells/well into a 6-well plate. After 24 h, the cells were treated with vehicle control, 5 μM DEX, 1 μM EPI or 1 μM NE for 48 h in a 5% CO_2 incubator at 37˚C. To quantify apoptosis, the treated cells were collected and stained with Annexin V-FITC and PI using an Annexin V/PI Apoptosis Detection Kit (Dojindo Laboratories, Inc.) according to the supplier's instructions as previously described (38,39). The apoptotic cells were then analyzed using a BD Accuri™ C6 Flow Cytometer (BD Biosciences) and built-in data analysis software (version 1.0.264.15; BD Biosciences).

Immunofluorescence staining. HepG2 cells were cultured on coverslips in a 6-well plate. After exposure to vehicle control, DEX, EPI or NE for 4 or 48 h, the cells were washed with PBS and fixed with 4% formaldehyde in PBS at room temperature for 15 min. The fixed cells were washed with 0.2% Triton X-100 at 4˚C for 2 min, incubated with ice-cold methanol at -20˚C for 10 min. After rinsing with PBS at room temperature for 5 min, the cells were blocked with 10% blocking serum (Beijing Solarbio Science & Technology Co., Ltd.) in PBS at 37˚C for 30 min. The cells were then probed with anti-γ-H2AX antibody (1:200 dilution) overnight at 4˚C followed by washing with PBS containing 1% BSA and incubation with FITC-conjugated goat anti-rabbit IgG (1:50 dilution; cat. no. ZF-0311; ZSGB Biotech) at room temperature for 1 h. After washing thrice with 1% BSA (cat. no. AP0027; NOVON Scientific) in PBS, the coverslips were incubated with 4',6-diamidine-2-phenylindole dihydrochloride (300 nM in PBS) for 30 min in the dark. The coverslips were then washed thrice with distilled water and mounted on slides before viewing with an Olympus DP72 microscope (Olympus Corp.).

Cycloheximide chase assay. A cycloheximide chase assay was performed to analyze protein stability as previously described (40). A total of $1x10^6$ HepG2 cells were plated in a 10-cm tissue culture dish. After 24 h, the cells were cotreated with 10 μg/ml cycloheximide (cat. no. C7698; MilliporeSigma) in the presence of DMSO vehicle control, 5 μM DEX, 1 μM EPI or 1 μM NE for 0, 6, 12, 24, 36, 48, 60 and 72 h in a 5% CO_2 incubator at 37˚C, followed by cell lysate preparation and western blotting analysis of Wip1 expression.

Western blotting. Western blotting analysis was performed to determine protein expression levels as previously described (41,42). Following exposure to vehicle control, DEX, EPI or NE, HepG2 cells were harvested and lysed using TNN buffer (50 mM Tris·HCl, pH 7.4; 150 mM NaCl; 20 mM EDTA; 0.5% NP-40; 1 mM Na_3VO_4; 50 mM NaF) with 2 mM PMSF and 1 mM dithiothreitol. After incubation on ice for 10 min and brief sonication, the cell lysates were centrifuged at 14,000 x g at 4˚C for 30 min and the supernatants were collected. The concentration of protein in the supernatant was quantified using a Bicinchoninic Acid Protein Assay Kit (Beijing Dingguo Changsheng Biotechnology Co., Ltd.). Equal amounts (40-80 μg) of the protein lysates were separated using 8-15% sodium dodecyl sulfate polyacrylamide gel electrophoresis. Then, the proteins were electroblotted onto polyvinylidene fluoride membranes (MilliporeSigma). The membranes were blocked in 5% non-fat milk in Tris-buffered saline containing 0.1% Tween-20 (TBST) for 60 min followed by incubation with primary antibodies against different proteins at 4˚C overnight. After washing with TBST three times, the membranes were

probed with secondary anti-IgG antibody conjugated with horseradish peroxidase [cat. no. 7074 (anti-rabbit) and 7076 (anti-mouse); 1:2,000 dilution; Cell Signaling Technology, Inc.] for 1 h at room temperature and visualized using an Efficient Chemiluminescence Kit (cat. no. GE2301-100ML; Beijing Dingguo Changsheng Biotechnology Co., Ltd.) with a Tanon Imaging System (Tanon Science & Technology Co., Ltd.). The relative protein levels were determined by measuring the intensity of the bands using Gel-Pro Analyzer software (version 4.0.00.001; Media Cybernetics, Inc.) and the target proteins were normalized against the internal control, GAPDH or β-actin.

Reverse transcription-quantitative polymerase chain reaction (RT-qPCR). Total RNA was extracted from the cultured cells using E.Z.N.A.® Total RNA Kit I (Omega Bio-Tek, Inc.) as described previously (43). The concentration of total RNA was measured using a NanoDrop ND-1000 spectrophotometer (NanoDrop Technologies; Thermo Fisher Scientific, Inc.). The synthesis of cDNA was performed using an All-In-One 5X RT MasterMix kit (Applied Biological Materials, Inc.) according to the manufacturer's recommendations. qPCR was then performed using EvaGreen® qPCR MasterMix (Applied Biological Materials, Inc.) on a StepOnePlus™ Real-Time PCR system (Applied Biosystems; Thermo Fisher Scientific, Inc.). The primers were used as follows: Wip1 forward, 5'-CTG TACTCGCTGGGAGTGAG-3' and reverse, 5'-GTTCGGGCT CCACAACGATT-3'; and GAPDH forward, 5'-AAGGAC TCATGACCACAGTCCAT-3' and reverse, 5'-CCATCACGC CACAGTTTCC-3'. GAPDH was used as the internal control. Thermocycling was initiated by a 10-min incubation at 95˚C, followed by 40 cycles of 95˚C for 15 sec and 60˚C for 1 min. The relative mRNA levels of Wip1 were determined using the $2^{-\Delta\Delta Cq}$ method (44).

Statistical analysis. Data are presented as the mean ± standard error of the mean. To assess the differences among groups, statistical analyses were performed by one-way analysis of variance (ANOVA) followed by Dunnett's post hoc test. For analyses with two independent variables, the data were analyzed by two-way ANOVA followed by Tukey's post hoc test. The analyses were performed using SPSS software (version 21; IBM Corp.). $P<0.05$ was considered to indicate a statistically significant difference.

Results

Stress hormones induce DNA damage in HepG2 human liver cancer cells. Exposure to stress or stress hormones has been reported to alter the genetic integrity of cancers by damaging their DNA and/or influencing the DNA maintenance machinery, specifically DNA damage response and DNA repair (20,21). To assess the DNA damage of HepG2 cells following exposure to stress hormones including cortisol and catecholamines, an alkaline comet assay was performed. The concentrations of DEX, EPI and NE were selected based on previous studies of stress hormone-mediated responses in HepG2 cells (45-49). A cytotoxicity assay also confirmed that the compounds were not cytotoxic at the concentrations used (Fig. S1). The alkaline comet assay was performed to quantify DNA damage after the exposure of HepG2 cells to 5 μM DEX, 1 μM EPI or 1 μM NE. A time-course experiment firstly verified that distinct DNA damage occurred 4 h following treatment with 1 μM NE (Fig. S2). When HepG2 cells were treated with 5 μM DEX for 4 h, the percentage of DNA in the comet's tail exhibited a statistically significant increase in DNA damage in response to DEX compared with vehicle-treated cells (32 vs. 6%; Fig. 1A and B). Treatment with 1 μM EPI or NE also induced similarly elongated tails compared with the vehicle control (38 and 28%, respectively; Fig. 1A and B). The DNA damage response persisted when the treatment duration was prolonged to 48 h (Fig. 1A and B). The DEX-treated HepG2 cells exhibited 2.7-fold more DNA damage compared with the DMSO vehicle-treated cells. Persistent DNA fragmentation was also observed in the EPI and NE-treated cells (1.6- and 3.0-fold higher than that in the vehicle-treated cells, respectively, although the increase was not significant for EPI). These results suggest that stress hormones induce DNA damage in HepG2 cells. To verify these findings, the level of γ-H2AX, an indicator of DNA damage, was evaluated by immunofluorescence in HepG2 cells following treatment with 5 μM DEX, 1 μM EPI or 1 μM NE for 4 and 48 h. The results showed increases in the punctate staining of γ-H2AX in the nuclei of HepG2 cells at 4 h after the stress hormone treatments compared with that in the vehicle-treated control cells (Fig. 1C). At 48 h after the treatments, the nuclear staining of γ-H2AX in the treated HepG2 cells remained at high levels (Fig. 1C). These observations are consistent with the findings of the comet assay.

HepG2 cells adapt to stress hormone-induced DNA damage. DNA damage activates DNA damage response factors which activate p53 to allow the accumulation of apoptotic proteins, thereby triggering cell death. In order to investigate this, a flow cytometric analysis was performed to quantify the proportion of apoptotic cells following treatment with DEX, EPI or NE. As shown in Fig. 2, no significant increase in apoptosis occurred in HepG2 cells after treatment with DEX, EPI and NE for 4 or 48 h when compared with the vehicle-treated control. These findings suggest that HepG2 cells adapt to the damage induced by stress hormones despite the presence of DNA lesions.

Stress hormones dephosphorylate DNA damage response factors by the upregulation of Wip1 in HepG2 cells. To identify how cancerous cells escape stress hormone-induced DNA stress, the levels of DNA damage response factors were determined in HepG2 cells following treatment with DEX, EPI or NE using western blotting. Fig. 3 show that the three stress hormones induced increments in the p-ATM (Ser1981)/ATM and p-Chk2 (Thr68)/Chk2 ratios compared with those in the cells treated with vehicle control following a 4-h treatment, indicative of the occurrence of stress hormone-induced DNA damage. These observations are consistent with the results of the comet assay. It is well established that Wip1 plays a vital role in stress signaling and the DNA damage response. When external stress induces DNA damage, Wip1 negatively regulates the DNA damage response via the dephosphorylation of its target proteins, including p53, MAPK and NF-κB. On the basis that stress hormones induce DNA damage instead

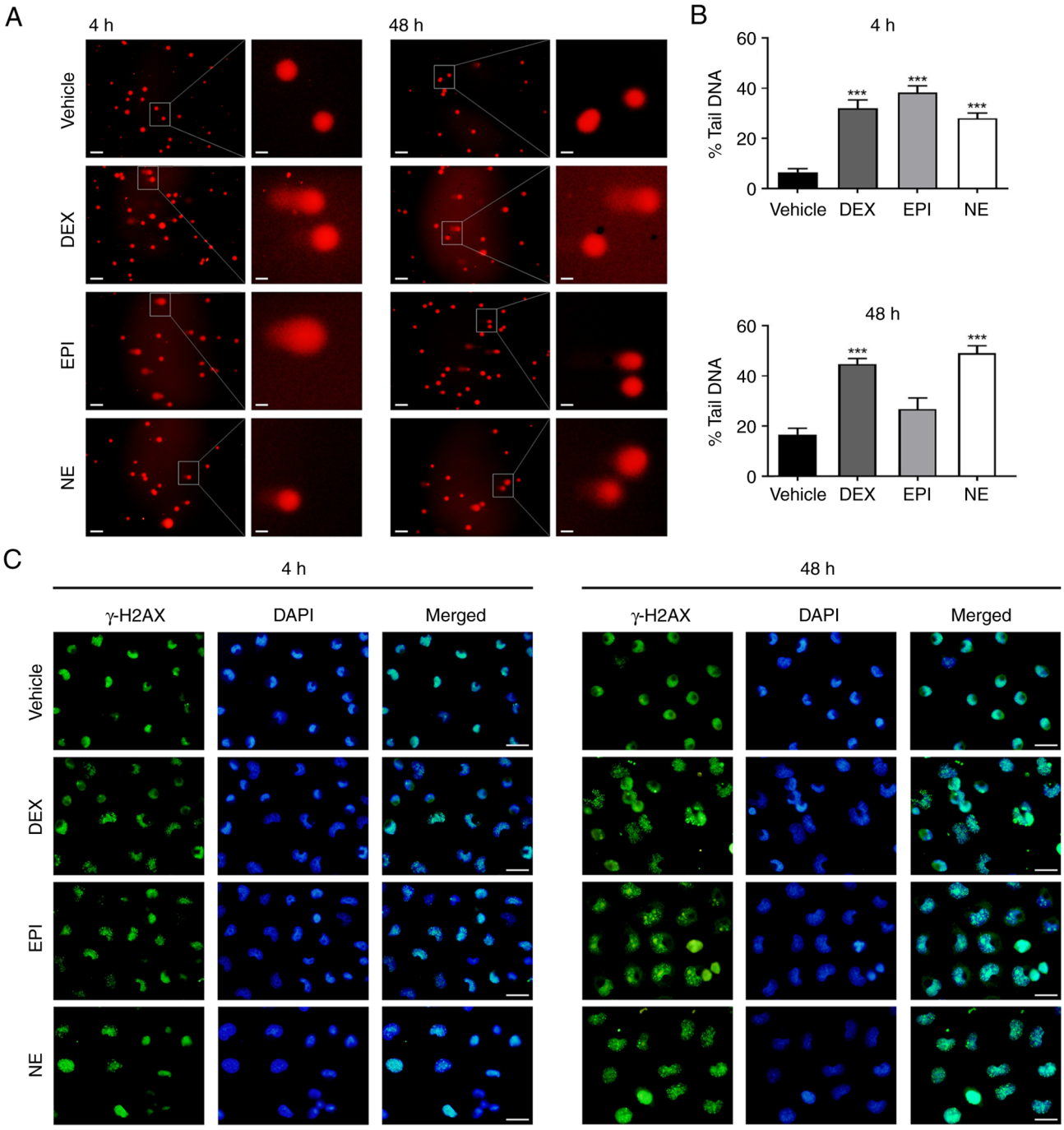

Figure 1. Stress hormones induce DNA damage in HepG2 cells. (A) Representative images of the comet assay. HepG2 cells were treated with DMSO vehicle control, 5 μM DEX, 1 μM EPI or 1 μM NE for 4 or 48 h and then subjected to an alkaline comet assay. Scale bar, 25 μm for main images and 5 μm for insets. (B) Histograms showing a quantitative analysis of the percentage of DNA in the comet tail after treatment with DEX, EPI or NE for 4 or 48 h. (C) Immunofluorescence staining of γ-H2AX in HepG2 cells following treatment with DMSO vehicle control, 5 μM DEX, 1 μM EPI or 1 μM NE for 4 or 48 h. Cell nuclei were stained with DAPI. Scale bar, 25 μm. ***P<0.001 vs. vehicle control. DMSO, dimethylsulfoxide; DEX, dexamethasone; EPI, epinephrine; NE, norepinephrine; γ-H2AX, phosphorylated histone 2AX.

of killing cells, we hypothesized that Wip1 negatively modulates the stress hormone-induced DNA damage response and allows tumor cells to adapt to the DNA damage. Therefore, the expression of Wip1 was examined following the various stress hormone treatments. As shown in Fig. 3, the expression of Wip1 increased following treatment with DEX, EPI or NE. The Wip1 expression level was observed to rise 48 h after DEX treatment. EPI induced a significant elevation at 4 h while NE significantly increased Wip1 expression after 4 h and further increased it at 48 h. However, the elevation of p-ATM and p-Chk2 levels was abrogated after 48 h. These findings suggest that Wip1 has a negative regulatory effect on stress hormone-induced DNA damage.

Stress hormones enhance Wip1 protein stability in HepG2 cells. To investigate the mechanism by which stress hormones upregulate Wip1, RT-qPCR was performed in HepG2 cells and the Wip1 mRNA level was observed to be unchanged

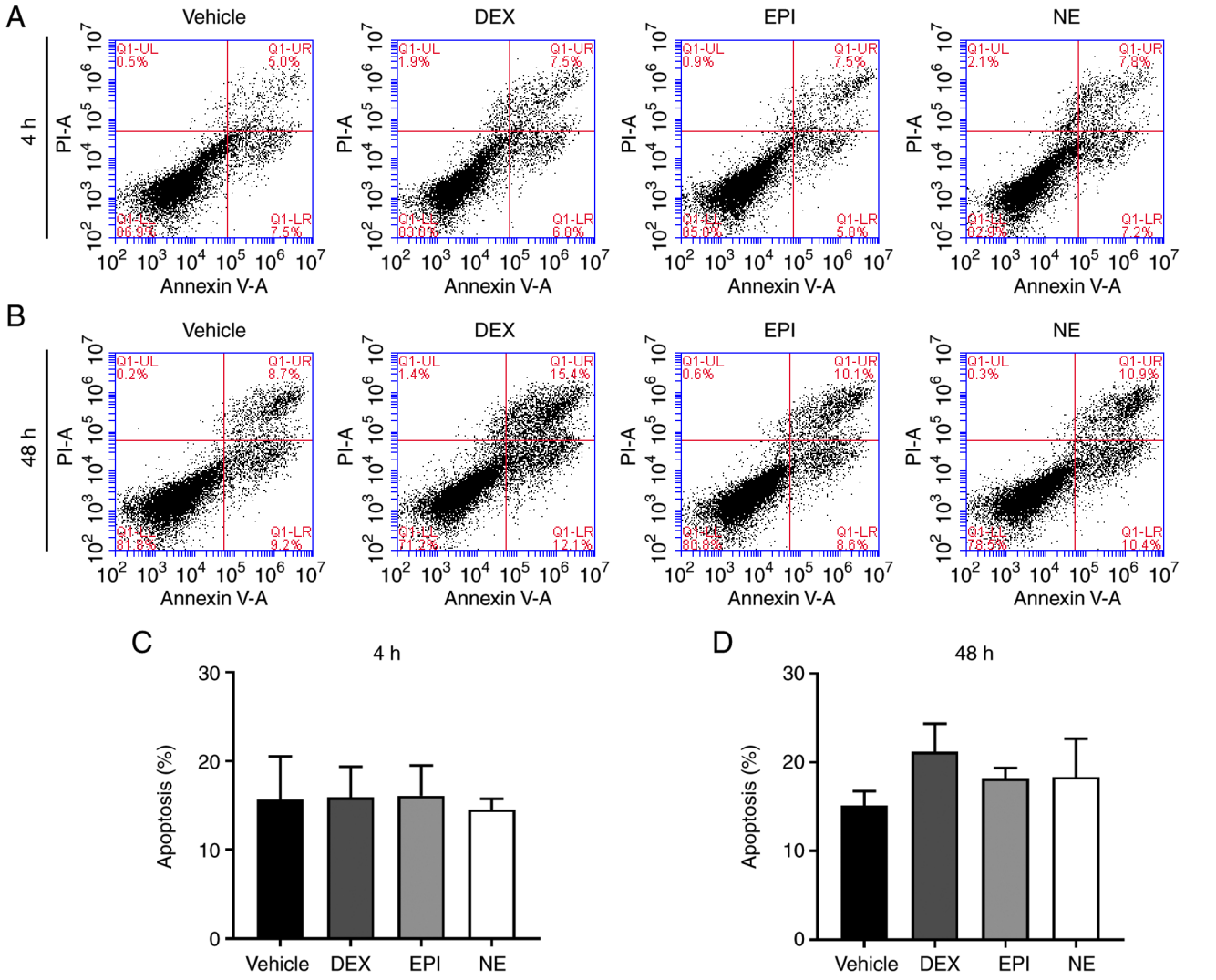

Figure 2. HepG2 cells escape stress hormone-induced DNA damage. Effects of 5 μM DEX, 1 μM EPI and 1 μM NE on the apoptosis of HepG2 cells after treatment for (A) 4 or (B) 48 h determined by staining apoptotic cells with Annexin V and PI followed by flow cytometric analysis. Bar charts showing the quantification of apoptosis data from three independent experiments after treatment for (C) 4 or (D) 48 h, including early apoptotic cells in the right lower quadrant and late apoptotic cells in the right upper quadrant. DEX, dexamethasone; EPI, epinephrine; NE, norepinephrine; PI, propidium iodide.

following stress hormone treatment (Fig. 4A). Since no significant change was detected in the Wip1 mRNA level following the treatment of HepG2 cells with DEX, EPI or NE for 48 h, we hypothesized that the mechanism of stress hormone-mediated Wip1 upregulation is a post-transcriptional process and examined the possibility that stress hormones maintain the stability of Wip1. To test this possibility, HepG2 cells were co-treated with cycloheximide, which is an inhibitor of elongation during protein synthesis, and DEX, EPI or NE; the effect on Wip1 protein stability was then determined. As is evident from Fig. 4B-E, the vehicle-treated cells exhibited a significant decline in Wip1 protein level from 24 h following cycloheximide treatment. However, when the HepG2 cells were co-treated with DEX and cycloheximide, Wip1 did not significantly decrease until 60 h after the start of treatment. The Wip1 protein level in the control cells was also lower than that of EPI- or NE-treated cells upon cycloheximide treatment while a significant loss of Wip1 in the cells co-treated with EPI or NE was observed at 72 or 60 h, respectively, following cycloheximide treatment. Overall, the loss of Wip1 in HepG2 cells treated with a combination of stress hormone and cycloheximide was much slower than that of the control treatment without stress hormones. This indicates that stress hormones maintain the stability of Wip1 protein.

Wip1 knockdown increases the levels of DNA damage response factors following treatment with stress hormones. To explore the role of Wip1 in the regulation of stress hormone-induced DNA damage, Wip1 protein expression in HepG2 cells was depleted using siRNA. Optimal transfection conditions were firstly identified via the use of different ratios of Wip1 siRNA and transfection reagents (Fig. S3). The siRNA-mediated knockdown of Wip1 in the HepG2 cells was demonstrated by the reduced expression level of Wip1 compared with that in cells transfected with control siRNA (Fig. 5A). Next, whether the loss of Wip1 affects stress hormone-induced DNA damage was examined via the immunoblotting of DNA damage response factors. Following treatment with stress hormones for 4 h, the Wip1-knockdown HepG2 cells presented elevated levels of γ-H2AX, a sensor of DNA double-strand breaks,

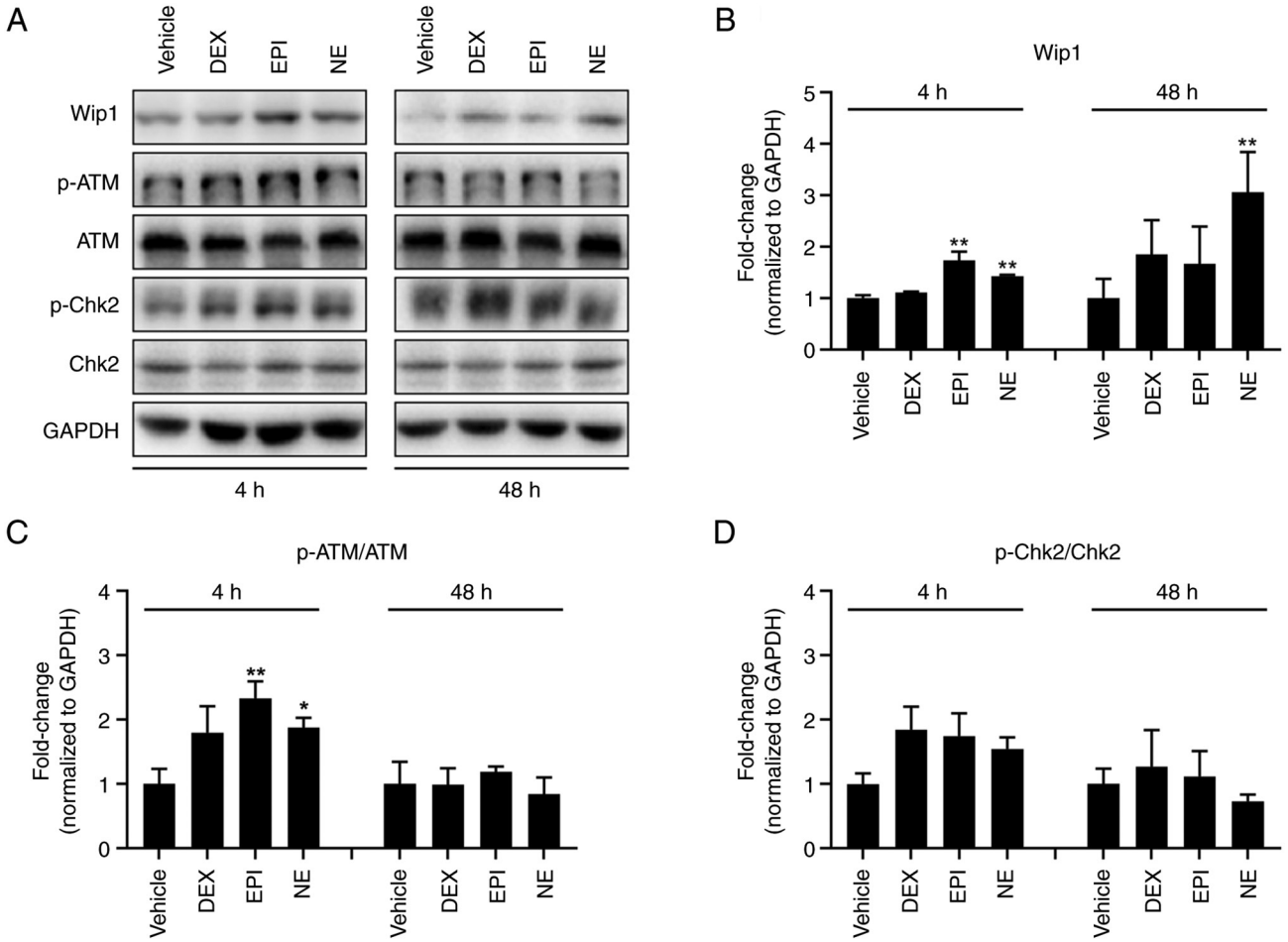

Figure 3. Stress hormones upregulate the Wip1 protein level in HepG2 cells. (A) Western blotting analysis of factors involved in the DNA damage response of HepG2 cells following treatment with DMSO vehicle control, 5 μM DEX, 1 μM EPI or 1 μM NE for 4 or 48 h. GAPDH was used as a loading control. Specific protein bands were determined according to their molecular weight. Note that the smearing and double banding of p-Chk2 may be due to low antibody specificity and future experiments may benefit from a change of antibody. Bar charts show quantitative data for (B) Wip1, (C) p-ATM and (D) p-Chk2 based on measurements of the density of the western blot bands and normalized against the GAPDH internal control or the total protein. *P<0.05 and **P<0.01 vs. the DMSO vehicle control. Wip1, wild-type p53-induced phosphatase 1; DMSO, dimethylsulfoxide; DEX, dexamethasone; EPI, epinephrine; NE, norepinephrine; p-, phosphorylated; ATM, ataxia-telangiectasia mutated; Chk2, checkpoint kinase 2.

indicating the occurrence of DNA damage (Figs. 5B and S4). When DNA damage occurs, the phosphorylation of Chk2 at Thr68 by ATM is attributable to the activation of Chk2 and downstream signaling (50-52). Also, DNA damage induces the phosphorylation p53 at Ser15 and Ser20, leading to cell cycle arrest, DNA repair or apoptosis (53,54). In the present study, the levels of p-p53 (Ser15) and p-Chk2 (Thr68) were also increased in Wip1-knockdown HepG2 cells, although the difference observed was not found to be significant due to high variation among the results of different experiments (Figs. 5B and S4). These findings suggest that Wip1 is involved in the DNA damage induced by stress hormones. To further verify the above findings, the level of γ-H2AX in HepG2 cells was determined by immunofluorescence 48 h after the knockdown of Wip1. The knockdown of Wip1 attenuated the punctate staining of γ-H2AX in the nuclei of HepG2 cells at 48 h after treatment compared with that in the respective cells transfected with control siRNA (Fig. 5C). Finally, the role of Wip1 in stress hormone-induced apoptosis was determined in Wip1-knockdown HepG2 cells following treatment with DEX, EPI or NE for 48 h. As is evident from Fig. 6, an increase in the proportion of apoptotic cells was observed in Wip1-knockdown HepG2 cells after stress hormone treatment, suggesting that Wip1 serves a role in the protection of HepG2 cells from stress hormone-induced apoptosis. However, this evidence requires further verification.

Discussion

In the present study, a DNA damage response was observed following the treatment of HepG2 cells with DEX, EPI and NE, but the induction of apoptotic cell death did not occur. Wip1, a type 2C family serine/threonine phosphatase, was found to contribute to the adaption of stress hormone-induced DNA damage by an upregulation in expression resulting from enhanced protein stability. The results of the present explorative study suggest that HepG2 cells may escape the stress hormone-induced DNA damage response via the suppression of DNA damage response factors. Ultimately, cancerous cells may take advantage of DNA damage adaptation to survive stimulation by chronic stress; however, the in-depth mechanism requires prospective validation.

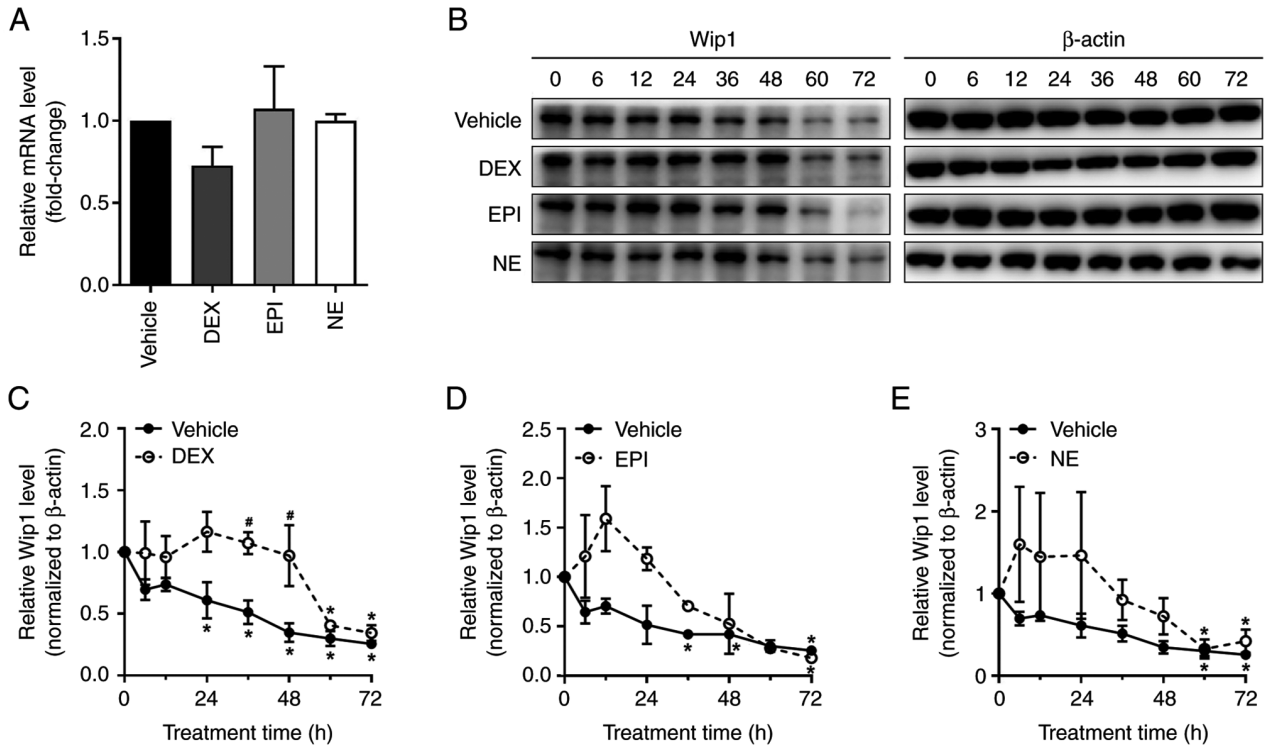

Figure 4. Stress hormones enhance the stability of Wip1 protein in HepG2 cells. (A) HepG2 cells were treated with DMSO vehicle control, 5 μM DEX, 1 μM EPI or 1 μM NE for 48 h and harvested for the analysis of Wip1 mRNA expression using reverse transcription-quantitative polymerase chain reaction. (B) HepG2 cells were treated with DMSO vehicle control, 5 μM DEX, 1 μM EPI or 1 μM NE for various time points in the absence or presence of 10 μg/ml cycloheximide and subjected to the analysis of Wip1 expression by western blotting. The Wip1 levels of the western blots for cells treated with (C) DEX, (D) EPI and (E) NE were determined by measuring the intensity of the bands using densitometric software, normalized against the internal control β-actin and plotted against the time of treatment. Data shown are the mean ± standard deviation of three independent experiments. *P<0.05 vs. 0 h; $^{\#}$P<0.05 vs. vehicle control at the corresponding time point. Wip1, wild-type p53-induced phosphatase 1; DMSO, dimethylsulfoxide; DEX, dexamethasone; EPI, epinephrine; NE, norepinephrine.

Cancer research has predominantly focused on investigations of gene mutations and signal transduction pathways in tumor cells, whereas the potential role of phycological stress has been less fully explored. The effects of stress hormones on tumor growth are mediated via the wide distribution of stress hormone receptors in cancer cells and the cellular signaling that occurs in response to the binding of stress hormones to their receptors. A variety of cancer cells such as liver, breast and ovarian cancer cells express receptors for glucocorticoids from the HPA axis and catecholamines from the SNS (55-57). The overexpression of glucocorticoid receptors has been identified as a potent survival pathway in breast cancer cells, and a study revealed that glucocorticoid receptor-mediated cancer cell survival signaling was activated by the administration of synthetic glucocorticoids as a premedication in chemotherapy treatment, which could potentially attenuate the effectiveness of chemotherapy (58). EPI and NE act as the physiological agonists for β-adrenergic receptors, with EPI preferentially binding to β_2-adrenergic receptors and NE binding with higher affinity to β_1-adrenergic receptors (59). Owing to the function of β-adrenergic receptors as G-protein-coupled cell membrane receptors, the psychological stress-stimulated release of EPI and NE triggers the formation of cAMP and activation of protein kinase A, leading to phosphorylation of the transcription factor cAMP response element binding protein (60). Similarly, glucocorticoids interfere with aberrant mechanisms in cancer cells by binding to glucocorticoid receptors, leading to nuclear translocation of the receptor-ligand complex, its binding to glucocorticoid receptor response elements in the promoter region of target genes and the activation of gene transcription. The activation of stress hormone receptors signals the stress from the extracellular environment to the tumor cell interior, leading to the growth and metastasis of malignant cancers (61). In the present study, it was observed that stress hormones enhanced the stability of Wip1 protein in HepG2 cells. Considering that no alteration in Wip1 mRNA expression was detected, we hypothesize that stress hormone-induced Wip1 upregulation occurs via post-transcriptional regulation induced by the activation of glucocorticoid or β-adrenergic receptors. However, further assessments of protein degradation pathways following stress hormone treatments would be helpful for determining the mechanism by which stress hormones regulate the stability of Wip1 and protect the protein from degradation.

It is essential to gain an improved understanding of how cancerous cells adapt to chronic stress for the translation of research to the clinic. In addition to transient effects such as heart rate changes or immune cell trafficking, the increased production of sympathetic and other adrenal hormones in response to stress causes long-lasting consequences such as permanent DNA damage, which results in increased cell transformation and/or tumorigenicity. Stress hormones can induce DNA damage sensors such as Chk1, Chk2 and E3 ubiquitin-protein ligase Mdm2, and the resulting DNA damage

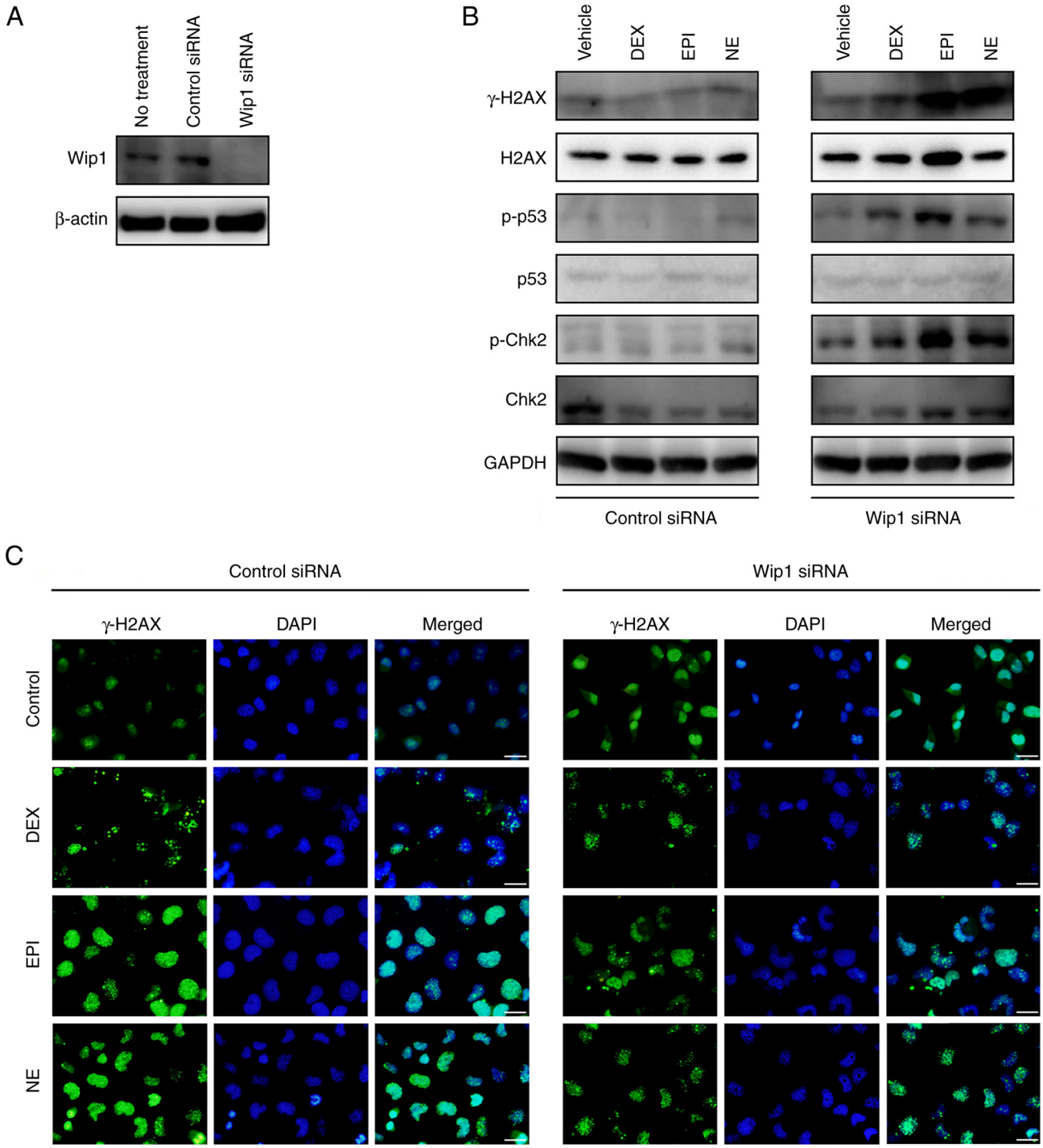

Figure 5. Wip1 knockdown cells are more sensitive to stress hormone-induced DNA damage response. (A) Western blotting assay of the Wip1 protein level in HepG2 cells transfected with control siRNA or Wip1 siRNA for 48 h. (B) Western blot analysis of DNA damage response factors in Wip1 knockdown and control cells following treatment with DMSO vehicle control, 5 μM DEX, 1 μM EPI or 1 μM NE for 4 h. GAPDH was used as a loading control. Specific protein bands were determined according to their molecular weight. Note that the smearing and double banding of p-Chk2 may be due to low antibody specificity and future experiments may benefit from a change of antibody. (C) Immunofluorescence staining of γ-H2AX in Wip1 knockdown and control HepG2 cells following treatment with DMSO vehicle control, 5 μM DEX, 1 μM EPI or 1 μM NE for 48 h. Cell nuclei were stained with DAPI. Scale bar, 25 μm. Wip1, wild-type p53-induced phosphatase 1; siRNA, small interfering RNA; DMSO, dimethylsulfoxide; DEX, dexamethasone; EPI, epinephrine; NE, norepinephrine; H2AX, histone 2AX; γ-H2AX, phosphorylated H2AX; p-, phosphorylated; Chk2, checkpoint kinase 2.

can trigger cellular responses, including cell cycle checkpoint activity, apoptosis and DNA repair pathways (62-64). However, few studies have explored the mechanism by which stress hormones impact cancer progression via the induction of DNA damage. In the present research, DNA fragmentation was observed after the treatment of human liver cancer cells with stress hormones for 4 h and persisted after 48 h of treatment with DEX and NE. It is noteworthy that there was no aggravation of EPI-induced DNA damage at 48 h. The suggestion that cells repair DNA breaks and continue to survive requires elucidation in future studies. However, overall, adaptation occurred, which is a process for allowing cell survival despite

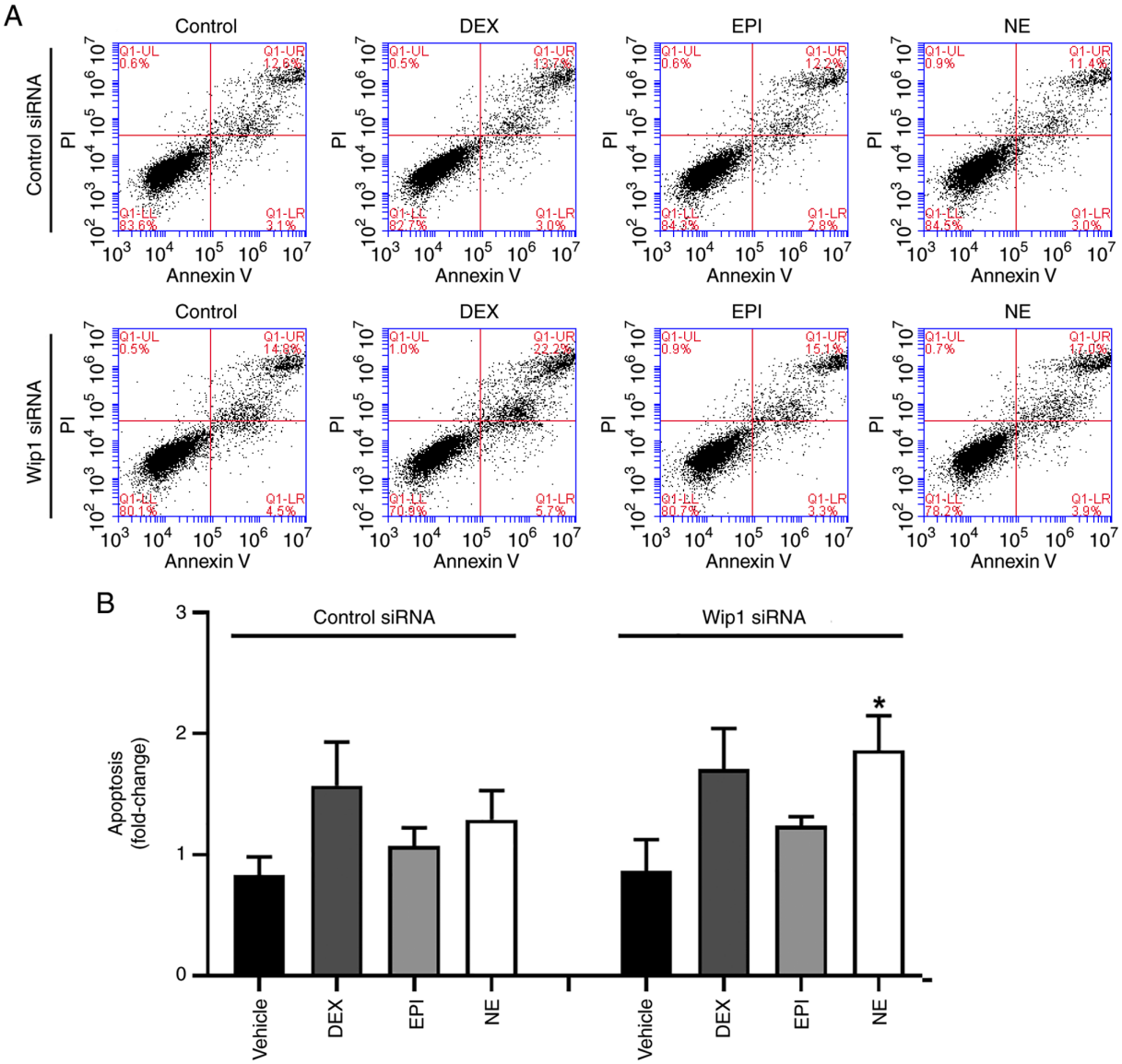

Figure 6. Knockdown of Wip1 promotes stress hormone-induced apoptosis. (A) Effects of 5 μM DEX, 1 μM EPI and 1 μM NE on the apoptosis of control and Wip1 knockdown HepG2 cells after 48 h assessed by staining apoptotic cells with Annexin V and PI followed by flow cytometric analysis. (B) Bar charts show the quantification of apoptosis data from four independent experiments, including early apoptotic cells in the right bottom quadrant and late apoptotic cells in the right upper quadrant. Fold change of apoptosis=apoptotic cells of the treatment group (%) ÷ apoptotic cells of the control group. *P<0.05 vs. vehicle control. Wip1, wild-type p53-induced phosphatase 1; siRNA, small interfering RNA; DEX, dexamethasone; EPI, epinephrine; NE, norepinephrine; PI, propidium iodide.

persistent DNA damage. The present study further identified the contribution of increased Wip1 protein stability to DNA damage adaption. The importance of Wip1 is evident from the fact that it is amplified and upregulated in liver, breast, ovarian, pancreatic, colorectal and gastric cancers (35,65-68). Within the DNA damage response mechanism, Wip1 acts as a homeostatic regulator via the dephosphorylation of important DNA damage sensor kinases, which facilitates the survival of tumor cells following the repair of DNA damage. Adaptation is thought to provide cancer cells with the opportunity to survive and undergo cell division with unrepaired DNA damage (69-71). However, the mechanism by which the accumulation of Wip1 promotes cancer progression in response to chronic stress requires more in-depth investigations. The relationship between Wip1 expression and stress hormones also merits further verification in patients with liver cancer.

Notably, the stress response is mediated by a complex and interconnected infrastructure constituted by the central and peripheral nervous systems, and cell-based research may not reflect the complex process that exists in the body. The detrimental effects of chronic stressors on tumor growth and progression are yet to undergo systematic evaluation in stress-based animal models. A few animal models are available for elucidating the etiology of stress-related tumor growth and metastasis, including the chronic restraint, social defeat and chronic unpredictable stress models. Repetitive exposure to these psychosocial or physical stresses mimics the features of human pathological conditions. Moreover, chronic stress exposure can be mimicked by treating rodents chronically with stress hormones. In our ongoing research, the chronic restraint stress model is being used to determine the role of Wip1 in stress-induced tumor immunosuppression. The identification

of the specific stress hormones and receptors involved in this process are likely to provide a fundamental understanding of the mechanisms by which the nervous system and the tumor tissue interact. More conclusive evidence and powerful findings could be obtained from additional studies using different cell lines and diverse animal models in order to minimize cell line specificity and the discrepancy between *in vitro* and *in vivo* conditions.

In conclusion, the present study provides preliminary evidence of the involvement of Wip1 in the tumor cell response to stress hormones. The results provide new insights into the mechanism underlying the effect of stress hormone signaling on DNA damage adaption. It is anticipated that the findings may be helpful in the development of more effective therapeutic interventions for the prevention and treatment of liver cancer, particularly in patients who are subjected to stress.

Acknowledgements

The authors would like to thank Dr Jingrong Cui (Peking University) for the gift of the human HepG2 cell line.

Funding

This study was primarily funded by the National Natural Science Foundation of China (grant no. 81972688). Additional support was provided by Peking University People's Hospital Scientific Research Developmental Funds (grant no. RDGS2022-03), the Beijing Municipal Natural Science Foundation (grant no. 7192128), the National Science and Technology Major Projects for Major New Drugs Innovation and Development (grant no. 2019ZX09301170) and the CAMS Innovation Fund for Medical Sciences (CIFMS; grant no. 2021-I2M-1-026).

Availability of data and materials

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

Authors' contributions

Conceptualization was performed by GL, WH and YL. Research methods were carried out by GL, WH, YQ, YC, MC, XY, DK, GW, HA and NY. Data analysis was performed by GL and WH. Administration was performed by NY. GL wrote the original draft of the manuscript, and WH, NY and YL reviewed and edited the manuscript. Funding was acquired by WH, HA and YL. Supervision was by YL. All authors read and approved the final version of the manuscript. GL, WH and YL confirm the authenticity of all the raw data.

Ethics approval and consent to participate

Not applicable.

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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